Developmental Biology

The Alk receptor tyrosine kinase regulates Sparkly, a novel activity regulating neurosecretory protein in the *Drosophila* CNS

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Abstract

Numerous roles for the Alk receptor tyrosine kinase have been described in *Drosophila*, including functions in the CNS, however the molecular details are poorly understood. To gain mechanistic insight, we employed Targeted DamID (TaDa) transcriptional profiling to identify targets of Alk signaling in the larval CNS. TaDa was employed in larval CNS tissues, while genetically manipulating Alk signaling output. The resulting TaDa data were analysed together with larval CNS scRNA-seq datasets performed under similar conditions, identifying a role for Alk in the transcriptional regulation of neuroendocrine gene expression. Further integration with bulk/scRNA seq and protein datasets from larval brains in which Alk signaling was manipulated, identified a previously uncharacterized *Drosophila* neuropeptide precursor encoded by *CG4577* as an Alk signaling transcriptional target. *CG4577*, which we named *Sparkly (Spar)*, is expressed in a subset of Alk-positive neuroendocrine cells in the developing larval CNS, including circadian clock neurons. In agreement with our TaDa analysis, overexpression of the *Drosophila* Alk ligand Jeb resulted in increased levels of Spar protein in the larval CNS. We show that Spar protein is expressed in circadian (Clock) neurons, and *Spar* mutant flies exhibit defects in sleep and circadian rhythm control. In summary, we report a novel activity regulating neuropeptide precursor gene that is regulated by Alk signaling in the *Drosophila* CNS.

eLife assessment

Receptor tyrosine kinases such as ALK play critical roles during appropriate development and behaviour and are nodal in many disease conditions, through molecular mechanisms that weren't completely understood. This manuscript identifies a previously unknown neuropeptide precursor as a downstream transcriptional target of Alk signalling in Clock neurons in the Drosophila brain. The experiments are well designed with attention to detail, the data are **solid** and the findings will be **useful** to those interested in events downstream of signalling by receptor tyrosine kinases.

Introduction

Receptor tyrosine kinases (RTK) are involved in wide range of developmental processes. In humans, the Anaplastic Lymphoma Kinase (ALK) RTK is expressed in the central and peripheral nervous system and its role as an oncogene in the childhood cancer neuroblastoma, which arises from the peripheral nervous system, is well described (Iwahara *et al*, 1997; Matthay *et al*, 2016; Umapathy *et al*, 2019; Vernersson *et al*, 2006).

In *Drosophila melanogaster,* ALK is expressed in the visceral mesoderm, central nervous system (CNS) and at neuromuscular junctions (NMJ). The critical role of *Drosophila* Alk and its ligand Jelly belly (Jeb) in the development of the embryonic visceral mesoderm has been extensively studied (Englund *et al*, 2003; Lee *et al*, 2003; Loren *et al*, 2003; Pfeifer *et al*, 2022; Popichenko *et al*, 2013; Schaub & Frasch, 2013; Shirinian *et al*, 2007; Varshney & Palmer, 2006; Wolfstetter *et al*, 2017). In the CNS, Alk signaling has been implicated in diverse functions, including targeting of photoreceptor axons in the developing optic lobes (Bazigou *et al*, 2007), regulation of NMJ synaptogenesis and architecture (Rohrbough & Broadie, 2010; Rohrbough *et al*, 2013) and mushroom body neuronal differentiation (Pfeifer *et al*., 2022). In addition, roles for Alk in neuronal regulation of growth and metabolism, organ sparing and proliferation of neuroblast clones, as well as sleep and long-term memory formation in the CNS have been reported (Bai & Sehgal, 2015; Cheng *et al*, 2011; Gouzi *et al*, 2011; Orthofer *et al*, 2020). The molecular mechanisms underlying these Alk-driven phenotypes are currently under investigation, and some potential molecular components of *Drosophila* Alk signaling in the larval CNS, such as the protein tyrosine phosphatase Corkscrew (Csw), have been identified in recent BioID-based *in vivo* proximity labeling analyses (Uckun *et al*, 2021).

In this work, we aimed to capture Alk-signaling dependent transcriptional events in the *Drosophila* larval CNS using Targeted DamID (TaDa) that profiles RNA polymerase II (Pol II) occupancy. TaDa employs a prokaryotic DNA Adenine methyltransferase (Dam) to specifically methylate adenines within GATC sequences present in the genome, creating unique $GA^{me}TC$ marks. In TaDa, Dam is fused to Pol II resulting in $GA^{me}TC$ marks on sequences adjacent to the Pol II binding site and can be combined with the Gal4/UAS system to achieve cell-type specific transcriptional profiling (Southall *et al*, 2013). Tissue specific TaDa analysis of Alk signaling, while genetically manipulating Alk signaling output, has previously been used to identify Alk transcriptional targets in the embryonic visceral mesoderm, such as the transcriptional regulator *Kahuli (Kah)* (Mendoza-Garcia *et al*, 2021). Here, we employed this strategy to identify Alk transcriptional targets in *Drosophila* larval brain tissue. These Alk TaDa identified transcripts were enriched in neuroendocrine cells. Further integration with bulk RNA-seq datasets generated from *Alk* gain-of-function and loss-of-function alleles, identified the uncharacterized neuropeptide precursor (*CG4577*), as an Alk target in the *Drosophila* brain, that we have named *Sparkly (Spar)*

based on its protein expression pattern. Spar is expressed in a subset of Alk-expressing cells in the central brain and ventral nerve cord, overlapping with the expression pattern of neuroendocrine specific transcription factor Dimmed (Dimm) (Hewes *et al*, 2003). Further, using genetic manipulation of Alk we show that Spar levels in the CNS respond to Alk signaling output, validating *Spar* as a transcriptional target of Alk. *Spar* mutant flies showed significant reduction in life-span, and behavioral phenotypes including defects in activity, sleep, and circadian rhythm. Notably, *Alk* loss-of-function alleles displayed similar behavioral defects, suggesting that Alk-dependant regulation of Spar in peptidergic neuroendocrine cells modulates activity and sleep/rest behavior. Interestingly, Alk and its ligand Alkal2 play a role in regulation of behavioral and neuroendocrine function in vertebrates (Ahmed *et al*, 2022; Bilsland *et al*, 2008; Borenas *et al*, 2021; Lasek *et al*, 2011a; Lasek *et al*, 2011b; Orthofer *et al*., 2020; Weiss *et al*, 2012; Witek *et al*, 2015). Taken together, our findings suggest an evolutionarily conserved role of Alk signaling in the regulation of neuroendocrine cell function and identify *Spar* as the first molecular target of Alk to be described in the regulation of activity and circadian clock in the fly.

Results

TaDa identifies Alk-regulated genes in *Drosophila* larval CNS

To characterize Alk transcriptional targets in the *Drosophila* CNS we employed Targeted DamID (TaDa). Briefly, transgenic DNA adenine methyl transferase (Dam) fused with RNA-Pol II (here after referred as Dam-Pol II) (Southall *et al*., 2013) (**Fig. 1A-B**), was driven using the pan neuronal *C155-Gal4* driver. To inhibit Alk signaling we employed a dominant negative Alk transgene, which encodes the Alk extracellular and transmembrane domain (here after referred as *UAS-Alk^{DN}*) (Bazigou *et al*., 2007) (**Fig. 1A**). Flies expressing Dam-Pol II alone in a wild-type background were used as control. Expression of Dam-Pol II was confirmed by expression of mCherry, which is encoded by the primary ORF of the TaDa construct (Southall *et al*., 2013) (**Fig. 1B, Fig. S1A-B'**). CNS from third instar wandering larvae were dissected and genomic DNA was extracted, fragmented at GA^{me}TC marked sites using methylation specific DpnI restriction endonuclease. The resulting GATC fragments were subsequently amplified for library preparation and NGS sequencing **(Fig. S1C)**. Bioinformatic data analysis was performed based on a previously described pipeline (Marshall & Brand, 2017; Mendoza-Garcia *et al*., 2021). Initial quality control analysis indicated comparable numbers of quality reads between samples and replicates, identifying >20 million raw reads per sample that aligned to the *Drosophila* genome **(Fig. S1D)**. No significant inter-replicate variability was observed **(Fig. S1E)**. Meta-analysis of reads associated with GATC borders showed a tendency to accumulate close to Transcription Start Sites (TSS) indicating the ability of TaDa to detect transcriptionally active regions **(Fig. S1F)**. A closer look at the Pol II occupancy profile of *Alk* shows a clear increase in Pol II occupancy from Exon 1 to Exon 7 (encoding the extracellular and transmembrane domain) in *Alk^{DN}* samples reflecting the expression of the dominant negative *Alk* transgene **(Fig. S1G)**.

To detect differential Pol II occupancy between Dam-Pol II control (*C155 Gal4>UAS-LT3-Dam::Pol II*) and *UAS-Alk^{DN}* (*C155-Gal4>UAS-LT3-Dam::Pol II; UAS-Alk^{DN}*) samples, neighbouring GATC associated reads, maximum 350 bp apart (median GATC fragment distance in the *Drosophila* genome) were clustered in peaks (Tosti *et al*, 2018). More than 10 million reads in both control and *Alk^{DN}* samples were identified as GATC associated reads **(Fig. S1D')**, and those loci displaying differential Pol II occupancy were defined by logFC and FDR (as detailed in materials and methods). Greater than 50% of aligned reads were in promoter regions, with 33.55% within a 1 kb range **(Fig. 1C, Table S1)**.

To further analyse transcriptional targets of Alk signaling we focused on loci exhibiting decreased Pol II occupancy when compared with controls, identifying 2502 loci with logFC<-2, FDR<0.05 **(Fig. 1D, Table S1)**. Genes previously known to be associated with Alk signaling, such as *kirre*,

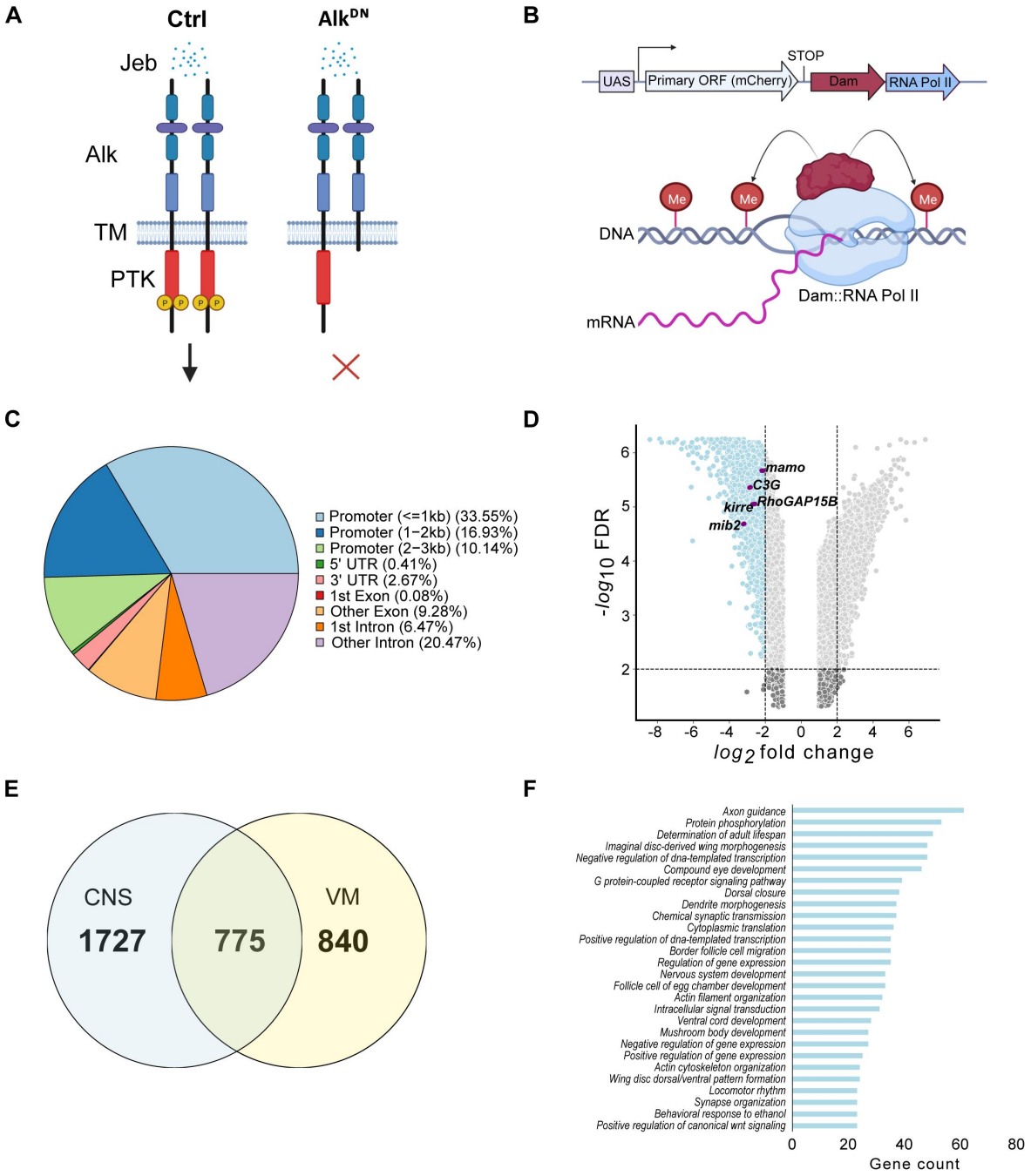


Figure 1.

TaDa-seq identifies novel Alk-regulated genes in the *Drosophila* larval CNS.

A. Schematic overview of experimental conditions comparing wild-type Alk (Ctrl) with Alk dominant-negative (Alk^{DN}) conditions. The *Drosophila* Alk RTK is comprised of extracellular, transmembrane and intracellular kinase (red) domains. Upon Jelly belly (Jeb, blue dots) ligand stimulation the Alk kinase domain is auto-phosphorylated (yellow circles) and downstream signaling is initiated. In Alk^{DN} experimental conditions, Alk signaling is inhibited due to overexpression of the Alk extracellular domain. **B.** The TaDa system (expressing *Dam::RNA Pol II*) leads to the methylation of GATC sites in the genome, allowing transcriptional profiling based on RNA Pol II occupancy. **C**. Pie chart indicating the distribution of TaDa peaks on various genomic features such as promoters, 5' UTRs, 3' UTRs, exons and introns. **D.** Volcano plot of TaDa-positive loci enriched in Alk^{DN} experimental conditions compared to control loci exhibiting Log2FC<-2, $p \geq 0.05$ are shown in blue. Alk-associated genes such as *mamo, C3G, Kirre, RhoGAP15B* and *mib2* are highlighted in purple. **E.** Venn diagram indicating Alk dependant TaDa downregulated genes from the current study compared with previously identified Alk-dependant TaDa loci in the embryonic VM (Mendoza-Garcia *et al*., 2021). **F.** Enrichment of Gene Ontology (GO) terms associated with significantly down-regulated genes in Alk^{DN} experimental conditions.

RhoGAP15B, *C3G*, *mib2* and *mamo*, were identified among downregulated loci (**Fig. 1D**). We compared CNS TaDa Alk targets with our previously published embryonic visceral mesoderm TaDa datasets that were derived under similar experimental conditions (Mendoza-Garcia *et al*., 2021) and found 775 common genes **(Fig. 1E, Table S1)**. Gene ontology (GO) analysis identified GO terms in agreement with previously reported Alk functions in the CNS (Bai & Sehgal, 2015; Bazigou *et al*., 2007; Cheng *et al*., 2011; Gouzi *et al*., 2011; Orthofer *et al*., 2020; Pfeifer *et al*., 2022; Rohrbough & Broadie, 2010; Rohrbough *et al*., 2013; Woodling *et al*, 2020) such as axon guidance, determination of adult lifespan, nervous system development, regulation of gene expression, mushroom body development, behavioral response to ethanol and locomotor rhythm (**Fig. 1F**). Many of the differentially regulated identified loci have not previously been associated with Alk signaling and represent candidates for future characterisation.

TaDa targets are enriched for neuroendocrine transcripts

To further characterise Alk-regulated TaDa loci, we set out to examine their expression in scRNA-seq data from wild-type third instar larval CNS (Pfeifer *et al*., 2022). Enrichment of TaDa loci were identified by using AUCell, an area-under-the curve based enrichment score method, employing the top 500 TaDa hits (Aibar *et al*, 2017) **(Fig. 2A-B, Table S1; Fig. S2)**. This analysis identified 786 cells (out of 3598), mainly located in a distinct cluster of mature neurons (**Fig. 2B, red circle; Fig. 2C**). This cluster was defined as neuroendocrine cells based on canonical markers, such as the neuropeptides *Lk* (*Leucokinin*), *Nplp1* (*Neuropeptide-like precursor 1*), *Dh44* (*Diuretic hormone 44*), *Dh31* (*Diuretic hormone 31*), *sNPF* (*short neuropeptide F*), *AstA* (*Allatostatin A*), and the enzyme *Pal2* (*Peptidyl-α-hydroxyglycine-α-amidating lyase 2*) as well as *Eip74EF* (*Ecdysone-induced protein 74EF*), and *Rdl* (resistance to dieldrin)(Guo *et al*, 2019; Huckesfeld *et al*, 2021; Takeda & Suzuki, 2022; Torii, 2009) (**Fig. 2D-F**). Overall, the TaDa-scRNAseq data integration analysis suggests a role of Alk signaling in regulation of gene expression in neuroendocrine cells.

To further explore the observed enrichment of Alk-regulated TaDa loci in neuroendocrine cells, we used a Dimmed (Dimm) transcription factor reporter (*Dimm Gal4>UAS-GFPcaax*), as a neuroendocrine marker (Park *et al*, 2008), to confirm Alk protein expression in a subset of neuroendocrine cells in the larval central brain and ventral nerve cord (**Fig. 2G**). This could not be confirmed at the RNA level, due to low expression of *dimm* in both our and publicly available single cell RNASeq datasets (Brunet Avalos *et al*, 2019; Michki *et al*, 2021; Pfeifer *et al*., 2022).

Multi-omics integration identifies *CG4577* as an Alk transcriptional target

Loci potentially subject to Alk-dependent transcriptional regulation were further refined by integration of the Alk-regulated TaDa dataset with previously collected RNA seq datasets (**Fig. 3A**). Specifically, w^{1118} (control), Alk^{Y1355S} (Alk gain-of-function) and $Alk^{\Delta RA}$ (Alk loss-of-function) RNA-seq datasets (Pfeifer *et al*., 2022) were compared to identify genes that exhibited both significantly increased expression in Alk gain-of function conditions (w^{1118} vs Alk^{Y1355S}) and significantly decreased expression in Alk loss-of-function conditions (w^{1118} vs $Alk^{\Delta RA}$ and control vs Alk^{DN}). Finally, we positively selected for candidates expressed in *Alk* positive cells in our scRNA-seq dataset. Notably, the only candidate which met these stringent criteria was *CG4577*, which encodes an uncharacterised putative neuropeptide precursor (**Fig. 3B**). *CG4577* exhibited decreased Pol II occupancy in Alk^{DN} samples **(Fig. 3C)**, and *CG4577* transcripts were upregulated in Alk^{Y1355S} gain-of-function conditions and downregulated in $Alk^{\Delta RA}$ loss-of-function conditions **(Fig. 3D)**. In agreement with a potential role as a neuropeptide precursor, expression of *CG4577* was almost exclusively restricted to neuroendocrine cell clusters in our scRNA-seq dataset (**Fig. 3E**). Examination of additional publicly available first instar larval and adult CNS scRNAseq datasets (Brunet Avalos *et al*., 2019; Davie *et al*, 2018) confirmed the expression of *CG4577* in Alk-expressing cells **(Fig. S3A-B)**. *CG4577-RA* encodes a 445 amino acid prepropeptide

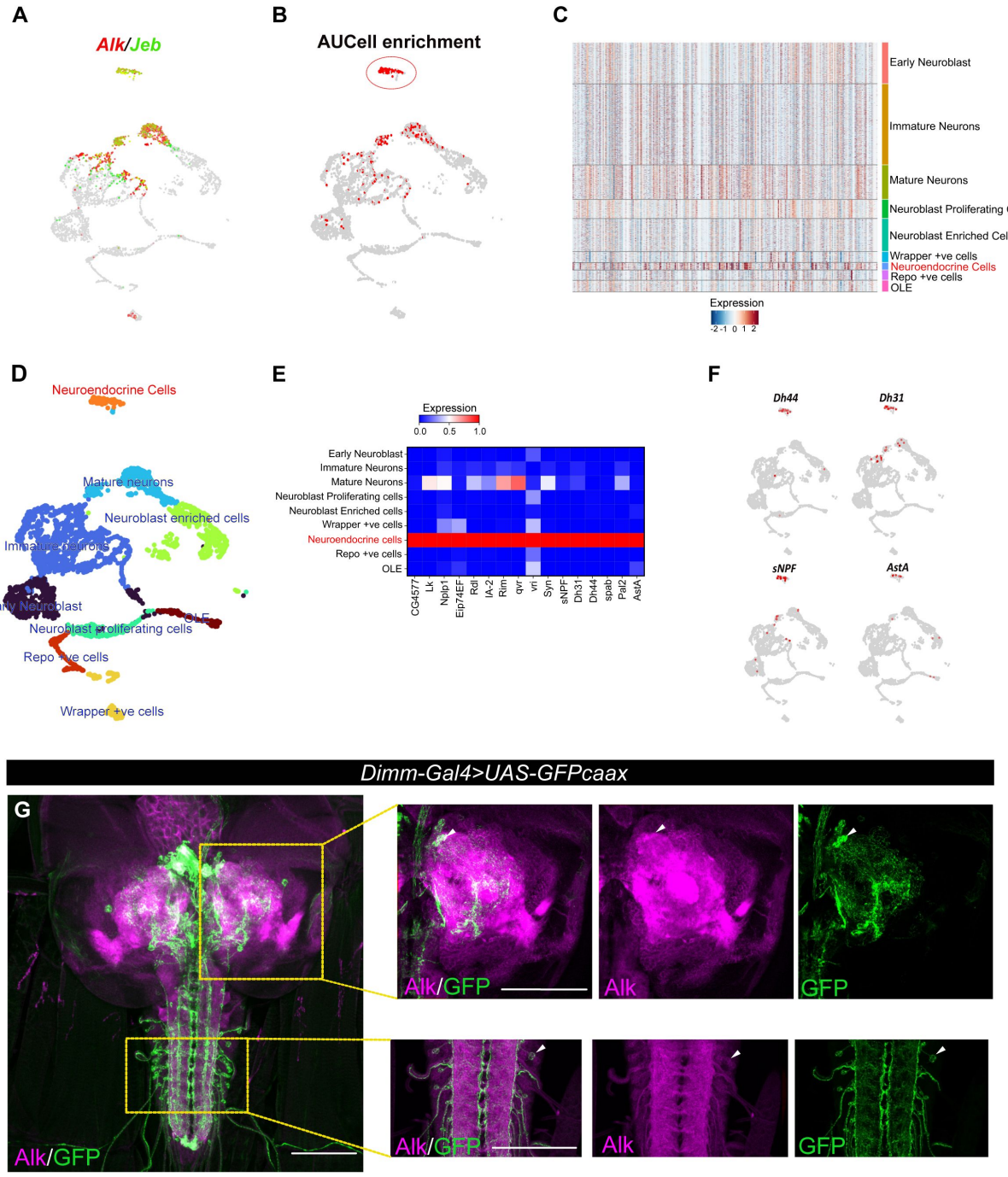


Figure 2.

Integration of TaDa data with scRNA-seq identifies an enrichment of Alk-regulated genes in neuroendocrine cells.

A. UMAP feature plot indicating Alk (in red) and Jeb (in green) mRNA expression in a control (w^{1118}) whole third instar larval CNS scRNA-seq dataset (Pfeifer *et al*., 2022). **B.** UMAP visualizing AUCell enrichment analysis of the top 500 TaDa downregulated genes in the third instar larval CNS scRNA-seq dataset. Cells exhibiting an enrichment (threshold >0.196) are depicted in red. One highly enriched cell cluster is highlighted (red circle). **C.** Heatmap representing expression of the top 500 genes downregulated in TaDa Alk^{DN} samples across larval CNS scRNA-seq clusters identifies enrichment in neuroendocrine cells. **D.** UMAP indicating third instar larval CNS annotated clusters (Pfeifer *et al*., 2022), including the annotated neuroendocrine cell cluster (in orange). **E.** Matrix plot displaying expression of canonical neuroendocrine cell markers. **F.** Feature plot visualizing mRNA expression of *Dh44*, *Dh31, sNPF* and *AstA* neuropeptides across the scRNA population. **G.** Alk staining in *Dimm-Gal4>UAS-GFPcaax* third instar larval CNS confirms Alk expression in Dimm-positive cells. Alk (in magenta) and GFP (in green), close-ups indicated by boxed regions and arrows indicating overlapping cells in the central brain and ventral nerve cord. Scale bars: 100 µm.

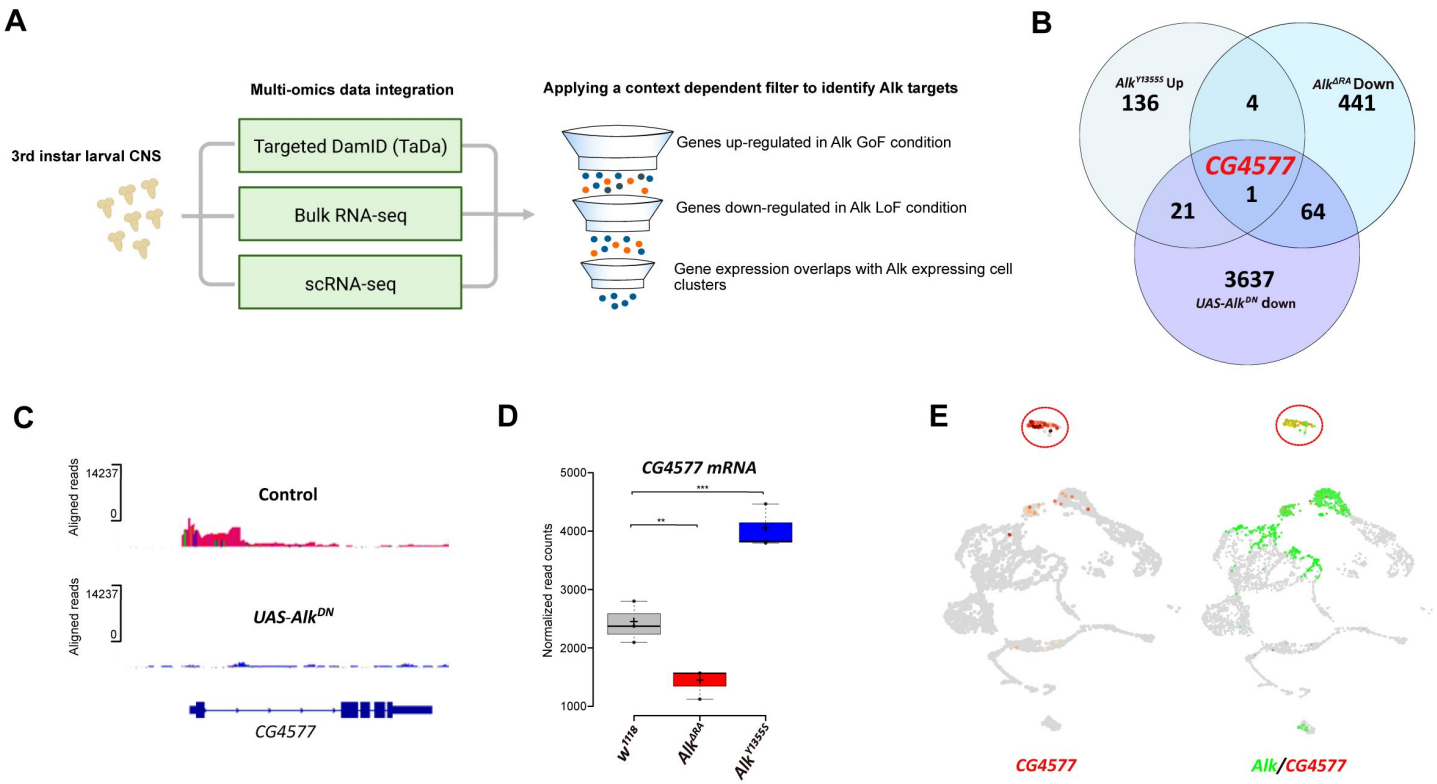
with a 27 aa N-terminal signal peptide sequence as predicted by SignalP-5.0) (**Fig. 3F**) (Almagro Armenteros *et al*, 2019). Analysis of CG4577-PA at the amino acid level identified a high percentage of glutamine residues (43 of 445; 9%), including six tandem glutamine repeats (amino acids 48-56, 59-62, 64-71, 116-118, 120-122 and 148-150) of unknown function as well as a lack of cysteine residues. The preproprotein has an acidic pI of 5.1 and carries a net negative charge of 6. Several poly- and di-basic prohormone convertase (PC) cleavage sites were also predicted (KR, KK, RR, RK) (Pauls *et al*, 2014; Southey *et al*, 2006; Veenstra, 2000) (**Fig. 3F**). Since the propeptide does not contain cysteine residues it is unable to form intracellular or dimeric disulfide bridges. A second transcript, *CG4577-RB*, encodes a 446 amino acid protein with only two amino acid changes **(Fig. S3C).** Phylogenetic analysis of *CG4577* relative to known *Drosophila* neuropeptide precursors failed to identify strong homology in keeping with the known low sequence conservation of neuropeptide prepropeptides outside the bioactive peptide stretches. However, we were also unable to find sequence homologies with other known invertebrate or vertebrate peptides. Next, we searched for *CG4577* orthologs across Metazoa. We obtained orthologs across the Drosophilids, Brachyceran flies and Dipterans. No orthologs were found at higher taxonomic levels, suggesting that *CG4577* either originated in Dipterans, or has a high sequence variability at higher taxonomic levels. To identify conserved peptide stretches indicating putative bioactive peptide sequences, we aligned the predicted aa sequences of the Dipteran *CG4577* orthologs. This revealed several conserved peptide stretches (**Fig. S4**) framed by canonical prohormone cleavage sites that might represent bioactive peptide sequences. BLAST searches against these conserved sequences did not yield hits outside of the Diptera.

CG4577/Spar is expressed in neuroendocrine cells

To further characterise *CG4577* we generated polyclonal antibodies that are predicted to recognize both CG4577-PA and CG4577-PB and investigated protein expression. CG4577 protein was expressed in a “sparkly” pattern in neurons of the third instar central brain as well as in distinct cell bodies and neuronal processes in the ventral nerve cord, prompting us to name CG4577 as Sparkly (Spar) (**Fig. 4A-B**). Co-labeling of Spar and Alk confirmed expression of Spar in a subset of Alk expressing cells, in agreement with our transcriptomics analyses (**Fig. 4A**). In addition, we also observed expression of Spar in neuronal processes which emerge from the ventral nerve cord and appear to innervate larval body wall muscle number 8, that may be either Leukokinin (Lk) or cystine-knot glycoprotein hormone GPB5 expressing neurons (**Fig. 4B**) (Cantera & Nässel, 1992; Sellami *et al*, 2011). Spar antibody specificity was confirmed in both *C155-Gal4>UAS-Spar-RNAi* larvae, where RNAi mediated knock down of *Spar* resulted in loss of detectable signal (**Fig. 4C-C**’), and in *C155-Gal4>UAS-Spar* larvae, exhibiting ectopic *Spar* expression in the larval CNS and photoreceptors of the eye disc (**Fig. 4D-D**’). To further address Spar expression in the neuroendocrine system, we co-labelled with antibodies to Dimm to identify peptidergic neuronal soma (Allan et al., 2005) in a *Dimm-Gal4>UAS-GFPcaax* background. This further confirmed the expression of Spar in Dimm-positive peptidergic neuroendocrine cells in the larval CNS (**Fig. 4E-E**”). Moreover, co-staining of Spar and Dimm in the adult CNS showed similar results **(Fig. S5)**.

Spar expression is modulated in response to Alk signaling activity

Our initial integrated analysis predicted *Spar* as a locus responsive to Alk signaling. To test this hypothesis, we examined Spar protein expression in w^{1118}, Alk^{Y1355S} and $Alk^{\Delta RA}$ genetic backgrounds, in which Alk signaling output is either upregulated (Alk^{Y1355S}) or downregulated ($Alk^{\Delta RA}$) (Pfeifer *et al*., 2022). We observed a significant increase in Spar protein in Alk^{Y1355S} CNS, while levels of Spar in $Alk^{\Delta RA}$ CNS appeared reduced but not significantly (**Fig. 4F-H**, quantified in **I**) (**Fig. 3D**). In agreement, overexpression of Jeb *(C155-Gal4>jeb)* significantly increased Spar levels when compared with controls *(C155-Gal4>UAS-GFPcaax)* (**Fig. 4J-L**, quantified in **M**). Again, while overexpression of dominant-negative Alk *(C155-Gal4>UAS-*Alk^{DN}*)*



F

CG4577-PA

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1-40    MPRISLPGSS LLVVLALILC LSLPIRAVPT SVLAQDTELS
41-80   TSANHKLQQQ QQQQQQHHQQ QQLQQQQQQQ QPATAKRSEE
81-120  ASAVPTADKK SSPEIVPASF SNAPSARNSP VPVSSQQQEQ
121-160 QQVPSGVYAL PSNEEILAAV AAAAQNSQQQ LQEEPSALEE
161-200 AVASSTMRKR GINYEYNPYS VASSDYGSDV PSGVWSDDYE
201-240 AAVPVSYGER DLQEIDDYVP ERRVSSSSAR NKAYDNLQNL
241-280 LNAEAYLESI PLSVPLTYAN RNYNVDERNK RGIYYNVGTP
281-320 GGNGASSLGS GSGYNDEGIN LNKYRRFNDM RLKRDTQLNP
321-360 ADMLALVALV EAGERARKES DAESSGPVPM IPSDDLDYAP
361-400 AGSWFDVPVQ ADYYGAGVPL DNQNQAMPKY EYVPRQHKYS
401-440 GVNSRFGSSK QRYMVAKKKR SVNQSQFMNE PVAERGSGYN
441-445 GEKYF
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Figure 3.

TaDa and RNA-seq identifies CG4577 as a novel Alk-regulated neuropeptide.

A. Flowchart representation of the multi-omics approach employed in the study and the context dependent filter used to integrate TaDa, bulk RNA-seq and scRNA-seq datasets. **B.** Venn diagram comparing bulk RNA-seq (Log2FC>1.5, p<0.05) and TaDa datasets (Log2FC<-2, p<0.05). A single candidate (*CG4577/Spar*) is identified as responsive to Alk signaling. **C.** TaDa Pol II occupancy of *CG4577/Spar* shows decreased occupancy in Alk^{DN} experimental conditions compared to control. **D.** Expression of *CG4577/Spar* in w^{1118} (control), $Alk^{\Delta RA}$ (*Alk* loss-of-function allele) and Alk^{Y1355S} (*Alk* gain-of-function allele) larval CNS. Box-whisker plot with normalized counts, ***p<0.01, *** p<0.01. **E.** Feature plot showing mRNA expression of *CG4577/Spar* and *Alk* in third instar larval CNS scRNA-seq data. Neuroendocrine cluster is highlighted (red circle). **F.** CG4577/Spar-PA amino acid sequence indicating the signal peptide (amino acids 1-26, in red), glutamine repeats (in green) and the anti CG4577/Spar antibody epitopes (amino acids 211-225 and 430-445, underlined).

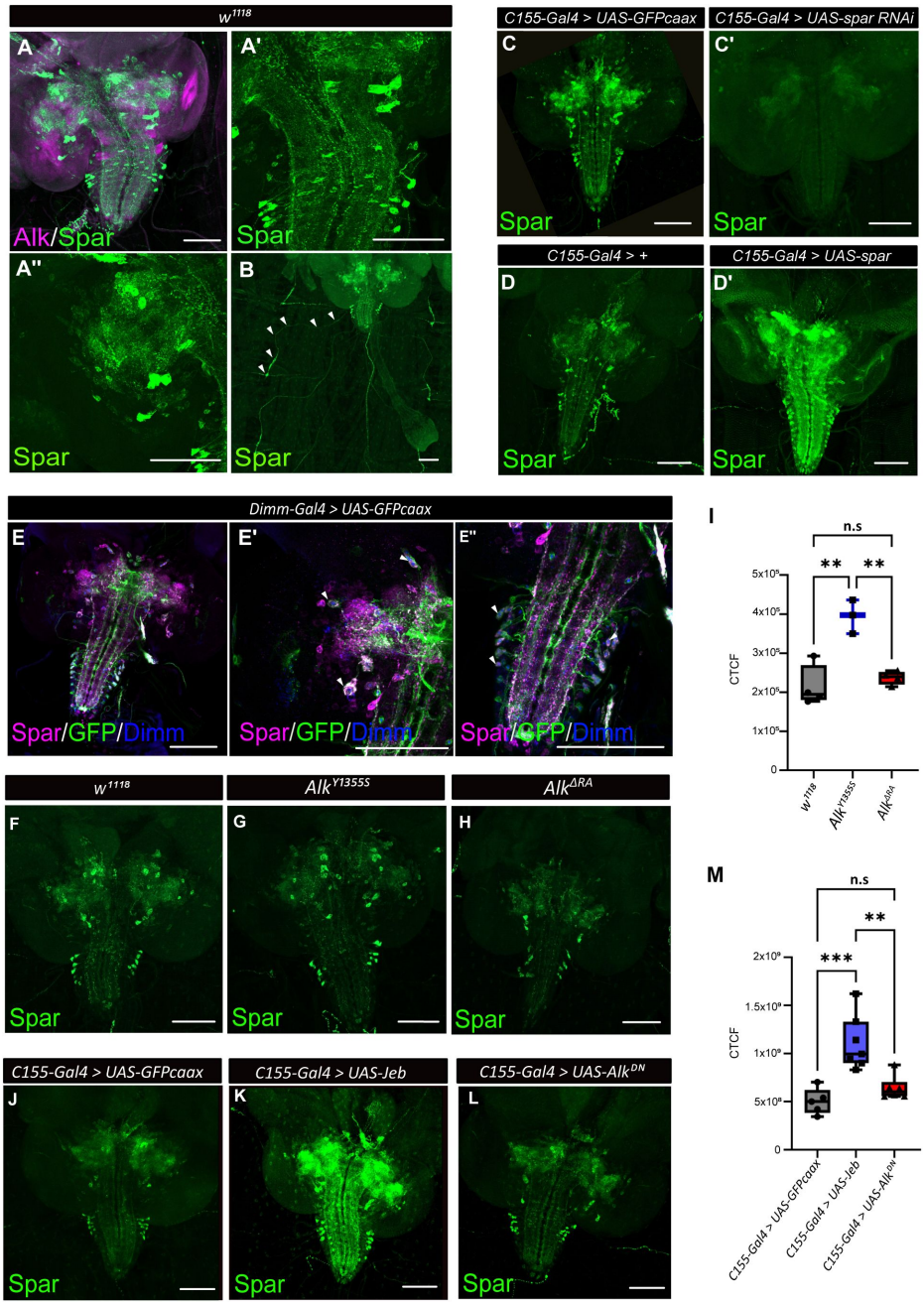

Figure 4.

Spar expression in the *Drosophila* larval brain.

A. Immunostaining of w^{1118} third instar larval brains with Spar (green) and Alk (magenta) revealing overlapping expression in central brain and ventral nerve cord. **A'-A".** Close-up of Spar expression (green) in central brain and ventral nerve cord respectively. **B.** Immunostaining of w^{1118} third instar larval CNS together with the body wall muscles, showing Spar (green) expression in neuronal processes (white arrowheads) which emerge from the ventral nerve cord and innervate larval body wall muscle number 8. **C-C'.** Decreased expression of Spar in third instar larval brains expressing *spar* RNAi (*C155-Gal4>Spar RNAi*) compared to control (*C155-Gal4>UAS-GFPcaax*) confirms Spar antibody specificity (Spar in green). **D-D'.** Spar overexpression (*C155 Gal4>UAS-Spar*) showing increased Spar expression (in green) compared to controls (*C155-Gal4>* +) larval CNS. **E-E".** Immunostaining of *Dimm-Gal4>UAS-GFPcaax* third instar larval brains with Spar (in magenta), GFP and Dimm (in blue) confirms Spar expression in Dimm-positive neuroendocrine cells (white arrowheads). **F-I.** Expression of Spar protein in third instar larval brains from w^{1118}, $Alk^{Y1355S,}$ and $Alk^{\Delta RA}$ genetic backgrounds. Spar expression is higher in Alk^{Y1355S} compared to w^{1118} controls, Spar levels quantified (corrected total cell fluorescence, CTCF) in **I**. **J-M.** Overexpression of Jeb in the third instar CNS (*C155-Gal4>UAS-jeb*) leads to increased Spar expression compared to controls (*C155-Gal4>UAS-GFPcaax*), Spar levels quantified (corrected total cell fluorescence, CTCF) in **M**. Scale bars: 100 μm.

appeared to result in decreased Spar levels, this was not significant (**Fig. 4L**, quantified in **M**). Taken together, these observations confirm that Spar expression is responsive to Alk signaling in CNS.

Spar encodes a canonically processed neurosecretory protein

To provide biochemical evidence for the expression of Spar, we re-analysed data from a previous peptidomic analysis of flies deficient for carboxypeptidase D (dCPD, SILVER) (Pauls *et al*, 2019), an enzyme that removes the basic C-terminal aa of peptides originating from PC cleavage of the proprotein. The analysis identified various peptides in non-digested extracts from genetic control brains (**Fig. 5**), including peptides framed by dibasic prohormone cleavage sequences, one of which (SEEASAVPTAD) was also obtained by *de-novo* sequencing (**Fig. 5**). This result demonstrates that the SPAR precursor is expressed and is processed into multiple peptides by PCs and possibly also other proteases. Analysis of the brain of *svr* mutant flies yielded similar results, but further revealed peptides C-terminally extended by the dibasic cleavage sequence (SEEASAVPTADKK, FNDMRLKR) (**Fig. 5**), thereby confirming canonical PC processing of the Spar propeptide. Of note, the phylogenetically most conserved peptide sequence of the Spar precursor (DTQLNPADMLALVALVEAGERA) framed by dibasic cleavage sites was among the identified peptides yet occurred only in control but not *svr* mutant brains (**Fig. 5**).

Additionally, we performed co-labeling with known *Drosophila* neuropeptides, Pigment-dispersing factor (PDF), Dh44, Insulin-like peptide 2 (Ilp2), AstA and Lk, observing Spar expression in subsets of all these populations (**Fig. 6**). These included the PDF-positive LNv clock neurons (**Fig. 6A-B**"), Dh44-positive neurons (**Fig. 6C- D**"), a subset of Ilp2 neurons in the central brain (**Fig. 6E-F**") and several AstA positive neurons in the central brain and ventral nerve cord (**Fig. 6G-H**"). We also noted coexpression in some Lk-positive neurons in the central brain and ventral nerve cord, that include the neuronal processes converging on body wall muscle 8 (**Fig. 6I- L**") (Cantera & Nässel, 1992). Similar Spar co-expression with PDF, Dh44, Ilp2, and AstA was observed in adult CNS (**Fig. S6**).

CRISPR/Cas9 generated *Spar* mutants are viable

Since previous reports have shown that Jeb overexpression in the larval CNS results in a small pupal size (Gouzi *et al*., 2011), we measured pupal size on ectopic expression of Spar (*C155-Gal4>Spar*) and *Spar RNAi* (*C155-Gal4>Spar RNAi*), noting no significant difference compared to controls (*C155-Gal4*>+ and *C155-Gal4>jeb*) **(Fig. S7)**. These results suggest that Spar may be involved in an additional Alk dependant function in the CNS. Further, experiments overexpressing Spar did not reveal any obvious phenotypes. To further investigate the function of Spar we generated a *Spar* loss of function allele by CRISPR/Cas9-mediated non-homologous end-joining, resulting in the deletion of a 716bp region including the *Spar* transcription start site and exon 1 (hereafter referred as $Spar^{\Delta Exon1}$) (**Fig. 7A**). Immunoblotting analysis indicated a 35kDa protein present in the wild-type (w^{1118}) controls that was absent in $Spar^{\Delta Exon1}$ mutant CNS lysates (**Fig. 7B**). The $Spar^{\Delta Exon1}$ mutant allele was further characterised using immunohistochemistry (**Fig. 7C-D**'). $Spar^{\Delta Exon1}$ shows a complete abrogation of larval and adult Spar expression, consistent with the reduction observed when *Spar* RNAi was employed (**Fig. 7C-D**'). $Spar^{\Delta Exon1}$ flies were viable, and no gross morphological phenotypes were observed, similar to loss of function mutants in several previously characterised neuropeptides such as Pigment dispersing factor (PDF), Drosulfakinin (Dsk) and Neuropeptide F (NPF) (Liu *et al*, 2019; Renn *et al*, 1999; Wu *et al*, 2020).

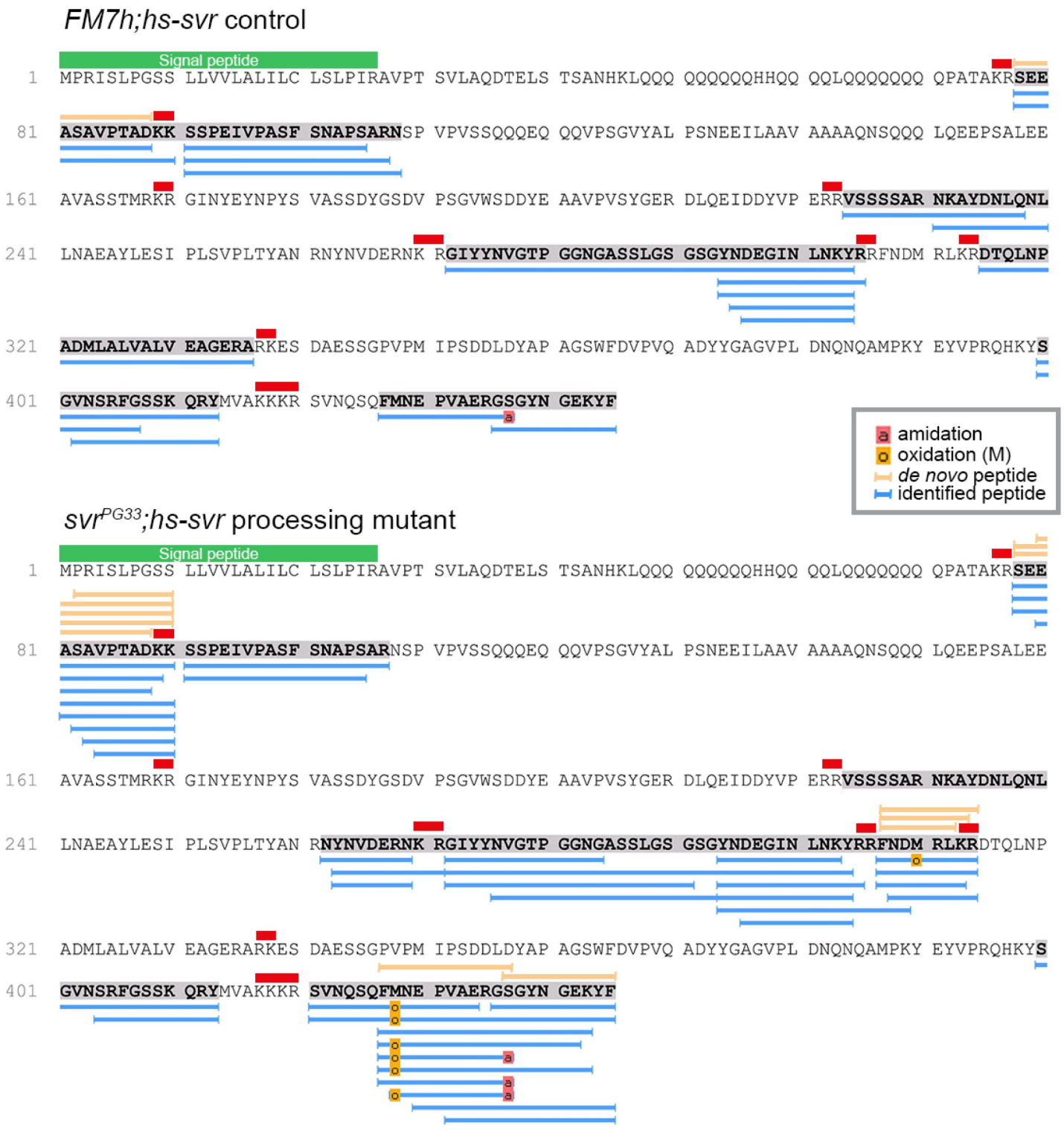

Figure 5.

Identification of Spar peptides in Drosophila CNS tissues.

Peptides derived from the Spar prepropeptide identified by mass spectrometry in wildtype-like control flies (*FM7h;hs-svr*, upper panel) and *svr* mutant (*svrPG33;hs-svr*, lower panel) flies. The predicted amino acid sequence of the CG4577-PA Spar transcript is depicted. Peptides identified by database searching (UniProt *Drosophila melanogaster*, 1% FDR) are marked by blue bars below the sequence. In addition, peptides correctly identified by *de novo* sequencing are marked by orange bars above the sequence. Red bars indicate basic prohormone convertase cleavage sites, green bar indicates the signal peptide.

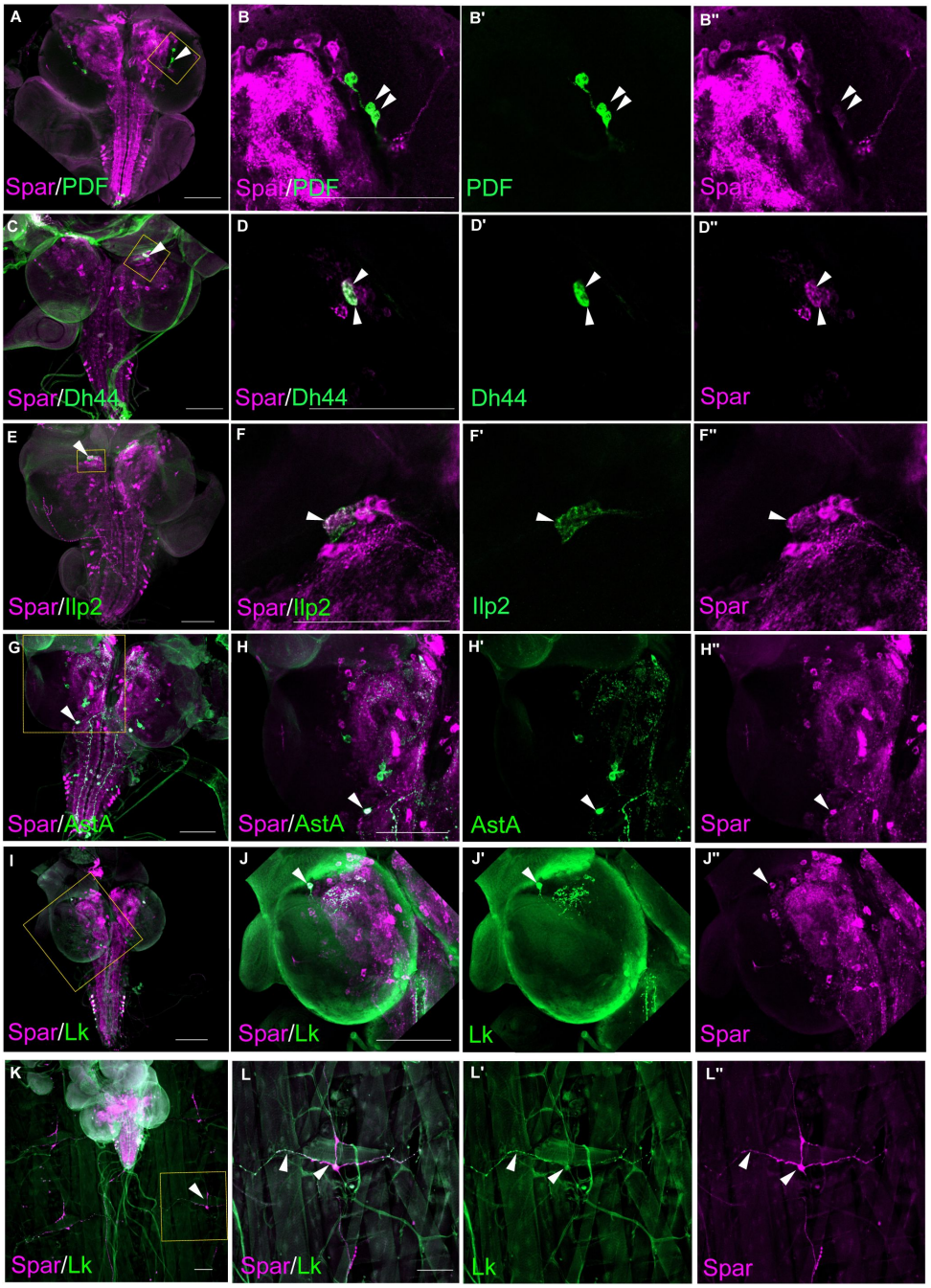

Figure 6.

Spar expression in larval neuropeptide expressing neuronal populations.

A. Immunostaining of w^{1118} third instar larval CNS with Spar (in magenta) and PDF (in green). Closeups (**B-B”)** showing PDF positive Spar neurons in central brain indicated by white arrow heads. **C.** Immunostaining of w^{1118} third instar larval CNS with Spar (in magenta) and Dh44 (in green). Closeups (**D-D”)** showing Dh44 positive Spar neurons in central brain indicated by white arrow heads. **E.** Immunostaining of w^{1118} third instar larval CNS with Spar (in magenta) and Ilp2 (in green). Closeups (**F-F”)** showing Ilp2 positive Spar neurons in central brain indicated by white arrow heads. **G.** Immunostaining of w^{1118} third instar larval CNS with Spar (in magenta) and AstA (in green). Closeups (**H-H”)** showing AstA positive Spar neurons in central brain indicated by white arrow heads. **I.** Immunostaining of w^{1118} third instar larval CNS with Spar (in magenta) and Lk (in green). Closeups (**J-J”)** showing Lk (LHLK neurons) positive Spar neurons in central brain indicated by white arrow heads. **K.** Immunostaining of w^{1118} third instar larval CNS together with the body wall muscles, showing Spar (in magenta) expressing Lk (in green) (ABLK neurons) in neuronal processes, which emerge from the ventral nerve cord and innervate larval body wall muscle. Closeups **(L-L”)** showing co-expression of Lk and Spar in neurons which attach to the body wall number 8 indicated by white arrow heads. Scale bars: 100 μm.

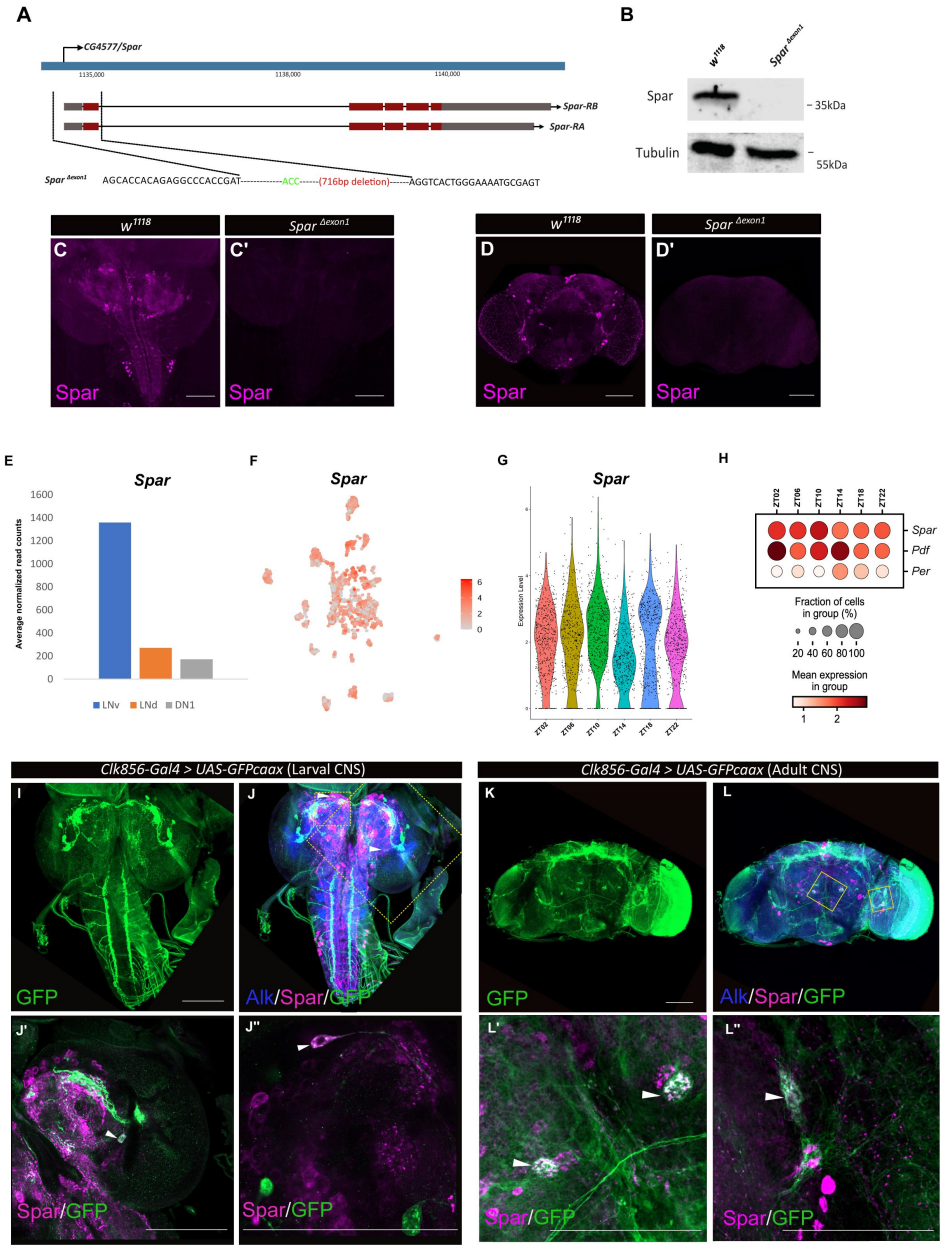

Figure 7.

Generation of *Spar*$^{\Delta Exon1}$ mutant and expression of *Spar* in circadian neurons.

A. Schematic overview of the *Spar* gene locus and the *Spar*$^{\Delta Exon1}$ mutant. Black dotted lines indicate the deleted region, which includes the transcriptional start and exon 1. **B.** Immunoblotting for Spar. Spar protein (35 kDa) is present in larval CNS lysates from wild-type (w^{1118}) controls but absent in *Spar*$^{\Delta Exon1}$ mutants **C-D'.** Immunostaining confirms loss of Spar protein expression in the *Spar*$^{\Delta Exon1}$ mutant. Third instar larval **(C-C')** and adult **(D-D')** CNS stained for Spar (in magneta). Spar signal is undetectable in *Spar*$^{\Delta Exon1}$. **E.** Expression of *Spar* in LNv, LNd and DN1 circadian neuronal populations, employing publicly available RNA-seq data (Abruzzi *et al*., 2017). **F.** Feature plot of *Spar* expression in circadian neurons, employing publicly available scRNA-seq data (Ma *et al*., 2021). **G.** Violin plot indicating *Spar* expression throughout the LD cycle, showing light phase (ZT02, ZT06 and ZT10) and dark phase (ZT14, ZT18 and ZT22) expression. **H.** Dotplot comparing *Spar* expression throughout the LD cycle with the previously characterized circadian-associated neuropeptide pigment dispersion factor (*Pdf*) and the core clock gene Period *(per)*. Expression levels and percentage of expressing cells are indicated. **I-J.** Spar expression in clock neurons (*Clk-Gal4>UAS-GFPcaax*) of the larval CNS **(I)**, visualized by immunostaining for Spar (magenta), Alk (in blue) and clock neurons (GFP, in green). **J'-J".** Close up of central brain regions (marked with yellow dotted box) indicating expression of Spar in clock-positive neurons (white arrowheads). **K-L.** Immunostaining of Clk-*Gal4>UAS-GFPcaax* in adult CNS with GFP (in green), Spar (in magenta) and Alk (in blue). **L'-L".** Close ups of CNS regions (marked with yellow dotted box regions) stained with GFP (in green) and Spar (in red) showing a subset of clock-positive neurons expressing Spar (white arrowheads).

Spar is expressed in a subset of clock-neurons in the larval and adult CNS

A previous report noted expression of *Spar* in the ventral lateral neuron (LNv), dorsal lateral neuron (LNd) and dorsal neuron 1 (DN1) populations of adult *Drosophila* circadian clock neurons (Abruzzi *et al*, 2017) (**Fig. 7E**). A meta-analysis of the publicly available single-cell transcriptomics of circadian clock neurons indicated that almost all adult cluster of clock neurons express *Spar* (Ma *et al*, 2021) (**Fig. 7F**). Additionally, we noted that the expression of *Spar* peaks around Zeitgeber time 10 (ZT10) (coinciding with the evening peak) (**Fig. 7G-H**), although the differences in expression level around the clock with LD or DD cycle were not dramatic **(Fig. S8A-C)**. To confirm the expression of Spar in circadian neurons at the protein level we co-stained Spar with a clock neuron reporter (*Clk856-Gal4>UAS-GFP*). A subset of Spar-positive larval CNS neurons appeared to be *Clk856-Gal4>UAS-GFP* positive (**Fig. 7I-J**"). Similarly, a subset of Spar-positive neurons in adults were GFP-positive (**Fig. 7K-L**"), confirming the expression of Spar protein in LNv clock neurons. Taken together, these findings suggest a potential function of the Alk-regulated TaDa-identified target Spar in the maintenance of circadian rhythm in *Drosophila*.

$Spar^{\Delta Exon1}$ mutants exhibit reduced adult lifespan, activity and circadian disturbances

Given the expression of Spar in circadian neurons of the larval CNS, and the previous observations of a role of Alk mutations in sleep dysregulation in flies (Bai & Sehgal, 2015), we hypothesised that $Spar^{\Delta Exon1}$ mutants may exhibit activity/circadian rhythm-related phenotypes. To test this, we first investigated the effects of loss of *Spar* (employing $Spar^{\Delta Exon1}$) and loss of *Alk* (employing a CNS specific loss of function allele of *Alk*, $Alk^{\Delta RA}$ (Pfeifer *et al*., 2022)) on adult lifespan and sleep/activity behaviour using the DAM (*Drosophila* activity monitor) system (Trikinetics Inc.). Both $Alk^{\Delta RA}$ and $Spar^{\Delta Exon1}$ mutant flies displayed a significantly reduced lifespan when compared to w^{1118} controls, with the $Spar^{\Delta Exon1}$ group exhibiting a significant reduction in survival at 25 days (**Fig. 8A**). Activity analysis in $Alk^{\Delta RA}$ and $Spar^{\Delta Exon1}$ flies under 12h light: 12h dark (LD) conditions indicated that both $Alk^{\Delta RA}$ and $Spar^{\Delta Exon1}$ flies exhibited two major activity peaks, the first centered around Zeitgeber time 0 (ZT0), the beginning of the light phase, the so-called morning peak, and the second around Zeitgeber time 12 (ZT12), the beginning of the dark phase that is called the evening peak (**Fig. 8B**, **black arrows**). Overall activity and sleep profiles per 24h showed increased activity in $Spar^{\Delta Exon1}$ flies (**Fig.8B-C**), that was more prominent during the light phase, with an increase in the anticipatory activity preceding both the night-day and the day-night transition in comparison to $Alk^{\Delta RA}$ and w^{1118} (**Fig. 8B**, **empty arrows**). The higher locomotor activity of $Alk^{\Delta RA}$ and $Spar^{\Delta Exon1}$ flies in comparison to wild-type was also visible in individual actograms (**Fig. 8D**). Furthermore, the mean activity and sleep over 30 days period of the experiment were also affected; the two mutant groups ($Alk^{\Delta RA}$ and $spar^{\Delta Exon1}$) displayed significant variations in activity and sleep means (**Fig. 8E-F**). Chi square rhythmicity analysis showed that $Alk^{\Delta RA}$ and w^{1118} in LD are more rhythmic compared to $Spar^{\Delta Exon1}$ flies **(Fig. S10A)**, however when comparing percentage of rhythmic flies among all groups the differences were not significant **(Fig. S9A')**. Since the defects observed in behaviour in both $Alk^{\Delta RA}$ and $Spar^{\Delta Exon1}$ mutants could be age dependent, we assessed mean activity and sleep after dividing the flies into two age groups "young" (1-12 days) and "old" (12-30 days). Notably, neither wake nor sleep activity was affected in old flies but was significantly perturbed in young flies (**Fig. 8G-H**). These results demonstrate that Spar is important for normal fly activity and loss of spar affects adult life cycle and sleep/wake activity.

Since $Spar^{\Delta Exon1}$ flies exhibited a hyperactive phenotype during both day and night hours, we sought to investigate a potential role of *Spar* in regulating the endogenous fly clock by assessing fly activity after shift to dark conditions. While control flies (**Fig. 9A-A**') adapted to the light-dark shift without any effect on mean activity and sleep (**Fig. 9A', D**'), $Spar^{\Delta Exon1}$ flies exhibited striking defects in circadian locomotor activity. Comparison of average activity and sleep during 5

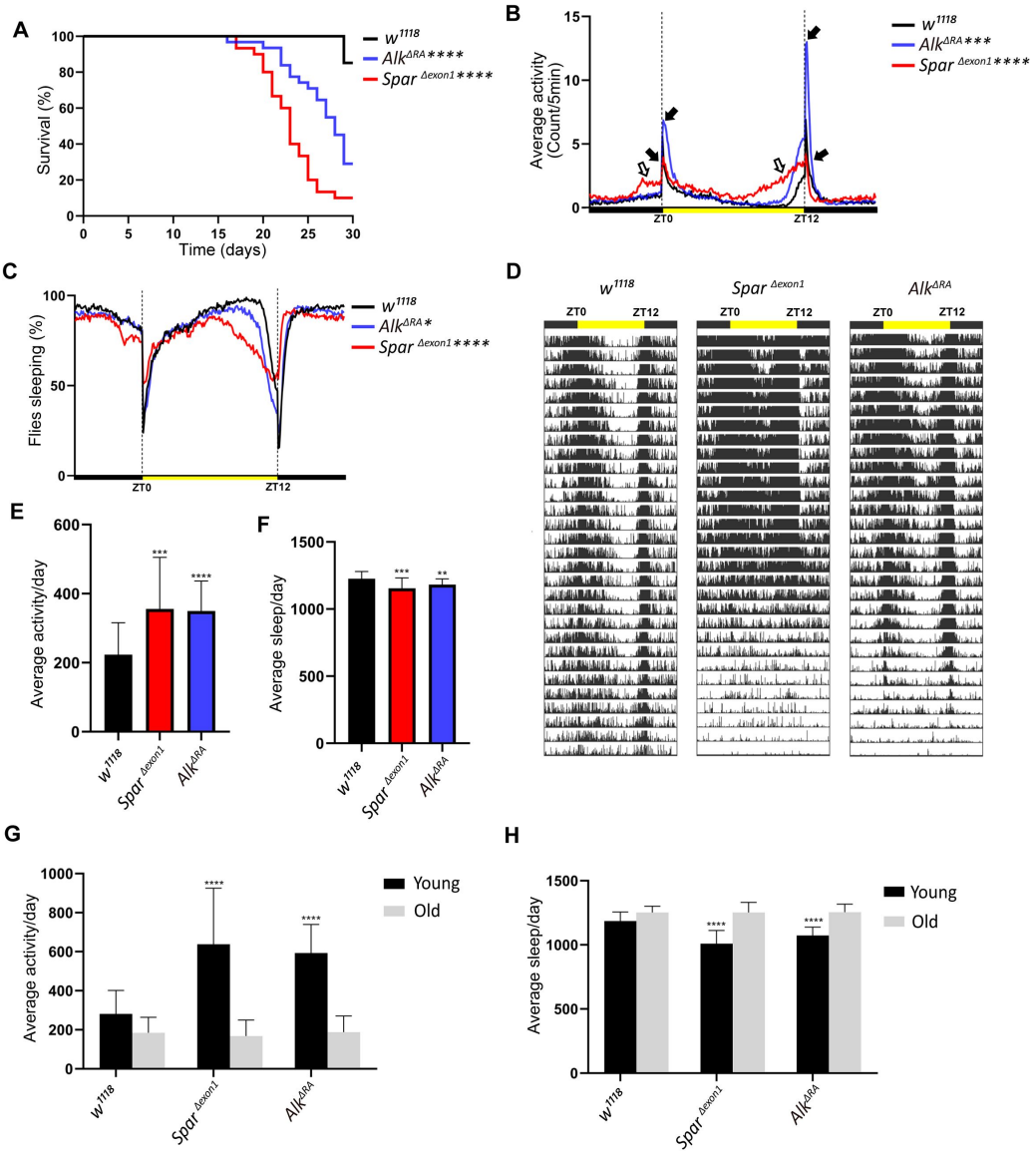

Figure 8.

Lifespan and activity plots of *Spar*$^{\Delta Exon1}$ mutants

A. Kaplan-Meier survival curve comparing *Alk*$^{\Delta RA}$ (n=31) and *Spar*$^{\Delta Exon1}$ (n=30) flies to *w*^{1118} controls (n=27). Outliers from each group were determined by Tukey's test, and statistical significance was analyzed by Log-rank Mantel-Cox test (****p<0.0001). **B.** Representative activity profile graph illustrating average activity count measured every 5 min across a 24-hour span. Black arrows indicate morning and evening activity peaks. Empty arrows indicate anticipatory increase in locomotor activity of *Spar*$^{\Delta Exon1}$ mutant flies occurring before light transition. An unpaired student t-test was used to determine the significance between wild-type and each mutant group (****p<0.0001; ***p<0.001). **C.** Representative sleep profile graph illustrating the percentage of time that flies spend sleeping measured every 5 min across a 24-hour span. An unpaired student t-test was used to determine the significance between wild-type and each mutant group (****p<0.0001; *p<0.05). **D.** Representative average actogram of the individual flies in each group. Each row corresponds to one day, visualized in 288 bars each representing one 5 min interval. Yellow bar represents the time of the day when the lights are turned on, with ZT0 indicating the morning peak and ZT12 the evening peak. **E.** Graph illustrating the mean locomotor activity per day across a 30-day span. An unpaired student t-test was used to determine the significance between wild-type and each mutant group (****p<0.0001; ***p<0.001). **F.** Graph illustrating the mean sleep per day across a 30-day span. An unpaired student t-test was used to determine the significance between wild-type and each mutant group (***p<0.001; **p<0.01). **G.** Graph illustrating the mean locomotor activity per day across a 30-day span. Flies were subdivided into two age groups (young:1-12 days and old: 13-30 days). An unpaired student t-test was used to determine the significance between wild-type and each mutant group for every age range (****p<0.0001). **H.** Graph illustrating the mean sleep per day across a 30-day span. Flies were subdivided into two age groups (young: 1-12 days and old: 13-30 days). An unpaired student t-test was used to determine the significance between wild-type and each mutant group for every age range (****p<0.0001; ***p<0.001; *p<0.05).

days of LD (light-dark) versus 5 days of DD (dark-dark) cycles, identified a reduction in mean activity under DD conditions in $Spar^{\Delta Exon1}$ flies (**Fig. 9B-B'**), consistent with an increase in average sleep mean (**Fig. 9E-E'**). Actogram profiling (**Fig. 9C**) showed that $Spar^{\Delta Exon1}$ flies exhibit a hyperactive profile consistent with our previous data in LD conditions, and maintain this hyperactivity when shifted into DD conditions (**Fig. 8D, S9A-B')**. Upon further in-depth examination of the activity profile in $Spar^{\Delta Exon1}$ we noticed that the transition to DD cycle resulted in a complete loss of anticipatory peaks with no activity peaks either at CT0 or at CT12 (**Fig. 9B, empty arrows**) and a dramatic change in activity and sleep bouts in these mutants (**Fig. 9B, E, S9**) which was not seen in control flies (**Fig. 9A, D**). The rhythmicity strength of $Spar^{\Delta Exon1}$ flies was similar between LD and DD conditions **(Fig. S10C)**, while control w^{1118} flies were less rhythmic in DD compared to LD conditions **(Fig. S10B)**. Both w^{1118} and $Spar^{\Delta Exon1}$ flies were rhythmic **(S10D-F)**.

Discussion

With the advent of multiple omics approaches, data integration represents a powerful, yet challenging approach to identify novel components and targets of signaling pathways. The availability of various genetic tools for manipulating Alk signaling in *Drosophila* along with previously gathered omics dataset provides an excellent basis for Alk centered data acquisition. We complemented this with TaDa transcriptional profiling allowing us to generate a rich dataset of Alk-responsive loci with the potential to improve our mechanistic understanding of Alk signaling in the CNS. A striking observation revealed by integrating our TaDa study with scRNAseq data was the enrichment of Alk-responsive genes expressed in neuroendocrine cells. These results are consistent with previous studies reporting expression of Alk in the *Drosophila* larval prothoracic gland (Pan & O'Connor, 2021), the neuroendocrine functions of Alk in mice (Ahmed *et al*., 2022; Reshetnyak *et al*, 2015; Witek *et al*., 2015) and the role of oncogenic Alk in neuroblastoma, a childhood cancer which arises from the neuroendocrine system (Matthay *et al*., 2016; Umapathy *et al*., 2019). In this study, we focused on one target of interest downstream of Alk, however, many additional interesting candidates remain to be explored. These include *CG12594*, *complexin* (*cpx*) and the *vesicular glutamate transporter* (*VGlut*) that also exhibit a high ratio of co-expression with *Alk* in scRNAseq data **(Fig. S2A)**.

Employing a strict context dependent filter on our integrated omics datasets identified Spar as a previously uncharacterized Alk regulated neuropeptide precursor. Spar amino acid sequence analysis predicts an N-terminal signal peptide and multiple canonical dibasic PC cleavage sites which are hallmarks of neuropeptide precursors. This is strong indication that Spar is shuttled to the secretory pathway and is post translationally processed within the Golgi or transport vesicles. Moreover, using mass spectrometry, we were able to identify predicted canonically processed peptides from the Spar precursor in undigested fly brain extracts. While all this points towards a neuropeptide-like function of Spar, other features appear rather unusual for a typical insect neuropeptide. First, the Spar propeptide is quite large for a neuropeptide precursor, and the predicted peptides do not represent paracopies of each other and do not carry a C-terminal amidation signal as is typical for *Drosophila* and other insect peptides (Nässel & Zandawala, 2019; Wegener & Gorbashov, 2008). Moreover, there are no obvious Spar or Spar peptide orthologues in animals outside the Diptera. We noted, however, that Spar is an acidic protein with a pI of 5.1 that lacks any cysteine residue. These features are reminiscent of vertebrate secretogranins, which are packaged and cleaved by PCs and other proteases inside dense vesicles in the regulated secretory pathway in neurosecretory cells (Helle, 2004). Secretogranins have so far not been identified in the *Drosophila* genome (Hart *et al*, 2017). Therefore, the identification of the neurosecretory protein Spar downstream of Alk in the *Drosophila* CNS is particularly interesting in light of previous findings, where VGF (aka secretogranin VII) has been identified as one of the strongest transcriptional targets regulated by ALK in both cell lines and mouse neuroblastoma models (Borenas *et al*., 2021; Cazes *et al*, 2014). *VGF* encodes a precursor

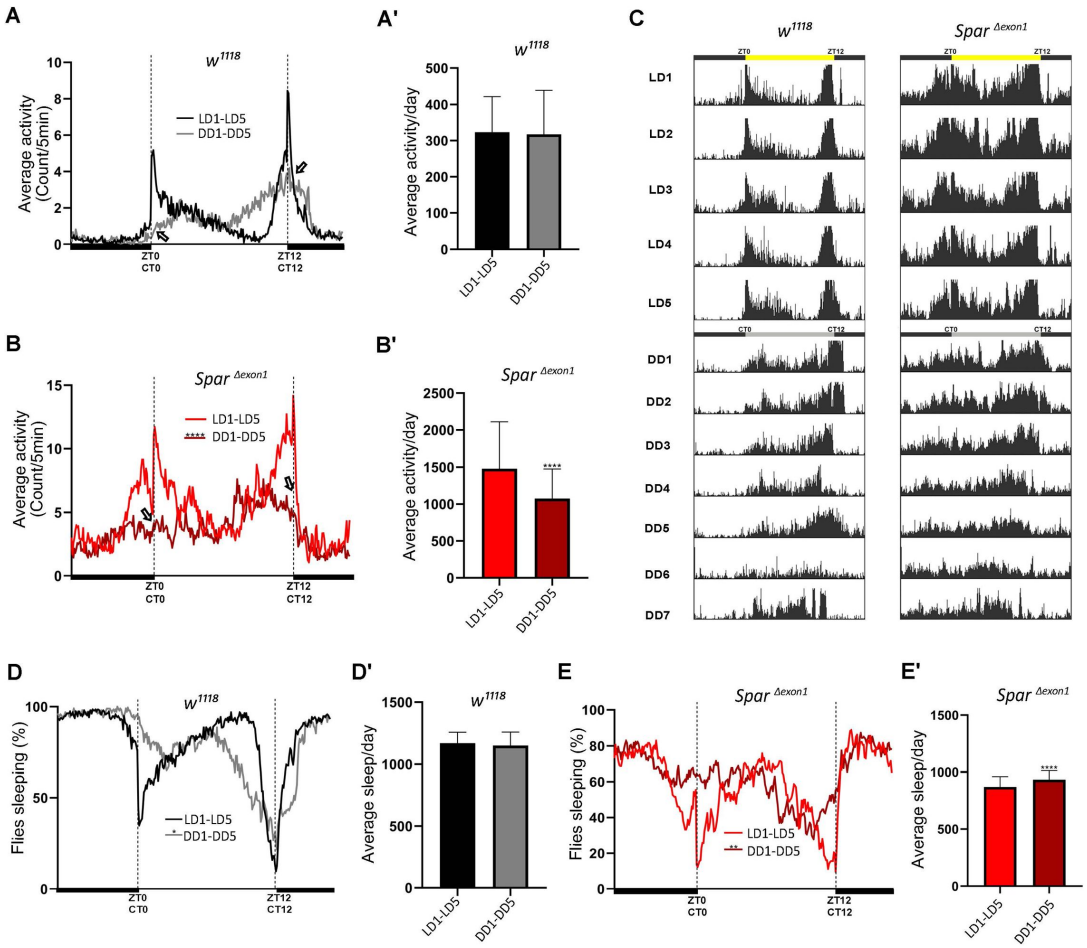

Figure 9.

Spar$^{\Delta Exon1}$ mutants exhibits circadian rhythm disturbances

A. Representative activity profile graph w^{1118} illustrating the average activity count measured every 5 min across a 24-hour span for Light-Dark (LD) for 5 cycles (black line) and subsequently switching to Dark-Dark (DD) for 5 cycles (gray lines). ZT0 and ZT12 represent the start and end of the photoperiod respectively. CT0 and CT12 represent the start and end of the constant dark conditions. Empty arrows indicate the morning and evening peaks at CT0 and CT12 respectively. A paired student t-test was used to determine the significance between the two experimental conditions. **A'.** Graph illustrating the mean locomotor activity per day of w^{1118} obtained by averaging 5 days in light/dark conditions (LD1-LD5) and 5 days in dark/dark conditions (DD1-DD5). A paired student t-test was used to determine the significance between the two experimental conditions. **B.** Representative activity profile graph of *Spar*$^{\Delta Exon1}$ illustrating the average activity count measured every 5 min across 24-hour span obtained by averaging 5 days in light/dark conditions (LD1-LD5) and 5 days in dark/dark conditions (DD1-DD5). Empty arrows indicate the morning and evening peaks at CT0 and CT12 respectively. A paired student t-test was used to determine the significance between the two experimental conditions (****p<0.0001). **B'.** Graph illustrating the mean locomotor activity per day of *Spar*$^{\Delta Exon1}$ obtained by averaging 5 days in light/dark conditions (LD1-LD5) and 5 days in dark/dark conditions (DD1-DD5). A paired student t-test was used to determine the significance between the two experimental conditions (****p<0.0001). **C.** Representative average actograms of the individual w^{1118} flies (n=32) and *Spar*$^{\Delta Exon1}$ flies (n=31) in LD and DD conditions. Each row corresponds to one day, visualized in 288 bars each representing one 5 min interval. ZT0 and ZT12 representing the start and end of the photoperiod respectively. CT0 and CT12 represent the start and end of the constant dark conditions. **D.** Representative sleep profile graph of w^{1118} illustrating the percentage of time that flies spend sleeping measured every 5 min across a 24-hour span obtained by averaging 5 days in light/dark conditions (LD1-LD5) and 5 days in dark/dark conditions (DD1-DD5). A paired student t-test was used to determine the significance between the two experimental conditions. (*p<0.05) **D'.** Graph illustrating the mean sleep per day of w^{1118} obtained by averaging 5 days in light/dark conditions (LD1-LD5) and 5 days in dark/dark conditions (DD1-DD5). A paired student t-test was used to determine the significance between the two experimental conditions. **E.** Representative sleep profile graph of *Spar*$^{\Delta Exon1}$ illustrating the percentage of time that flies spend sleeping measured every 5 min across a 24-hour span obtained by averaging 5 days in light/dark conditions (LD1-LD5) and 5 days in dark/dark conditions (DD1-DD5). A paired student t-test was used to determine the significance between the two experimental conditions (****p<0.0001). **E'.** Graph illustrating the mean sleep per day of *Spar*$^{\Delta Exon1}$ obtained by averaging 5 days in light/dark conditions (LD1-LD5) and 5 days in dark/dark conditions (DD1-DD5). A paired student t-test was used to determine the significance between the two experimental conditions (****p<0.0001).

polypeptide, which is processed by PCs generating an array of secreted peptide products with multiple functions that are not yet fully understood at this time (Lewis *et al*, 2015; Quinn *et al*, 2021).

Using a newly generated antibody we characterized the expression of Spar in the *Drosophila* CNS, showing that its expression overlaps with the Dimm transcription factor that is expressed in the fly neuroendocrine system (Hewes *et al*., 2003), suggesting that Spar is expressed along with multiple other neuropeptides in pro secretory cells of the CNS (Park *et al*., 2008). Spar is also expressed in well-established structures such as the mushroom bodies, which are known to be important in learning and memory and regulate food attraction and sleep (Joiner *et al*, 2006; Pitman *et al*, 2006), and where Alk is also known to function (Bai & Sehgal, 2015; Gouzi *et al*., 2011; Pfeifer *et al*., 2022). Interestingly, Spar is expressed in a subset of peptidergic neurons which emerge from the ventral nerve cord (VNC) and innervate larval body wall muscle number 8. In larvae these Lk-expressing neurons of the VNC, known as ABLKs, are part of circuitry that regulates locomotion and nociception, and in adults they regulate water and ion homeostasis (Imambocus *et al*, 2022; Okusawa *et al*, 2014; Zandawala *et al*, 2018). The role of Spar in this context is unknown and requires further investigation. The identity of the Spar receptor, as well as its location, both within the CNS and without, as suggested by the expression of Spar in neurons innervating the larval body wall is another interesting question for a future study. In our current study we focused on characterising Spar in the *Drosophila* CNS. To functionally characterise Spar in this context we generated null alleles with CRISPR/Cas9 and investigated the resulting viable $Spar^{\Delta Exon1}$ mutant.

Spar transcript expression in *Drosophila* clock neurons has been noted in a previous study investigating neuropeptides in clock neurons, however Spar had not been functionally characterised at the time (Abruzzi *et al*., 2017, Ma *et al*, 2021). We have been able to show that Spar protein is expressed in clock neurons of the larval and adult CNS, findings that prompted us to study the effect of Spar in activity and circadian rhythms of flies. *Drosophila* activity monitoring experiments with $Spar^{\Delta Exon1}$ and Alk loss of function ($Alk^{\Delta RA}$) mutants revealed striking phenotypes in life span, activity and sleep. In *Drosophila* a number of genes and neural circuits involved in the regulation of sleep have been identified (Shafer & Keene, 2021). The role of Alk in sleep has previously been described in the fly, where Alk and the Ras GTPase Neurofibromin1 (Nf1), function together to regulate sleep (Bai and Sehgal, 2015). Indeed, a study in mice has reported an evolutionarily conserved role for Alk and Nf1 in circadian function (Weiss *et al*, 2017). While these studies place Alk and Nf1 together in a signaling pathway that regulates sleep and circadian rhythms, no downstream effectors transcriptionally regulated by the Alk pathway have been identified that could explain its regulation of *Drosophila* sleep/activity. Our data suggest that one way in which Alk signaling regulates sleep is through the control of Spar, as $Spar^{\Delta Exon1}$ mutants exhibit a striking activity phenotype. The role of clock neurons and the involvement of circadian input in maintenance of long term memory (LTM) involving neuropeptides such as PDF has been previously described (Inami *et al*, 2022). Since both Alk and Nf1 are also implicated in LTM formation in mushroom body neurons (Gouzi, Bouraimi et al 2018), the potential role of Nf1 in Spar regulation and the effect of Spar loss on LTM will be interesting to test in future work. It can be noted that both insulin producing cells (IPCs) and DH44 cells of the pars intercerebralis and the Lk producing LHLK neurons of the brain and certain AstA neurons in the brain are involved in regulation of aspects of metabolism and sleep (Barber *et al*, 2021; Cavey *et al*, 2016; Chen *et al*, 2016; Cong *et al*, 2015; Donlea *et al*, 2018; Nässel & Zandawala, 2022; Yurgel *et al*, 2019). Furthermore, DH44 cells of pars intercerebralis in S **Fig 6 C,D** are major players in regulation feeding and courtship in adults (Barber *et al*., 2021; Cavanaugh *et al*, 2014; Dus *et al*, 2015; King *et al*, 2017; Oh *et al*, 2021). In conclusion, our TaDa analysis identifies a role for Alk in regulation of endocrine function in *Drosophila*. These results agree with the previously reported broad role of Alk in functions such as sleep, metabolism, and olfaction in the fly and in the hypothalamic-pituitary-gonadal axis and Alk-

driven neuroblastoma responses in mice. Finally, we identify *Spar* as the first neuropeptide precursor downstream of Alk to be described that regulates activity and circadian clock function in the fly.

Materials and methods

Drosophila stocks and Genetics

Standard *Drosophila* husbandry procedures were followed. Flies were fed on Nutri Fly® Bloomington Formulation food (Genesee Scientific, Inc.) cooked according to the manufacturer's instruction. Crosses were reared at 25°C. The following stocks were obtained from Bloomington *Drosophila* Stock Center (BDSC): w^{1118} (BL3605), *Dimm- Gal4* (also known as *C929-Gal4*) (BL25373), and *C155-Gal4* (BL458). Additional stocks used in this study are the following: *UAS-LT3-NDam-Pol II* (Southall *et al*., 2013), *UAS-Alk*^{DN} (*P{UAS-Alk.EC.MYC}* (Bazigou *et al*., 2007)), *UAS-Jeb* (Varshney & Palmer, 2006), Alk^{Y1335S} (Pfeifer *et al*., 2022), $Alk^{\Delta RA}$ (Pfeifer *et al*., 2022), $Spar^{\Delta Exon1}$ (this study), *UAS-Spar* (this study) and *UAS-Spar*^{2xOLLAS} (this study).

TaDa Sample preparation

Pan neuronal *C155-Gal4* expressing animals were crossed with either *UAS-LT3 Dam::Pol II* (Control) or *UAS-LT3-Dam::Pol II; UAS-Alk*^{EC} (Alk dominant negative sample) and crosses were reared at 25°C. Approximately 100-150 third instar larval brains were dissected in cold PBS for each technical replicate. Genomic DNA was extracted using QIAGEN blood and tissue DNA extraction kit and methylated DNA was processed and amplified using previously described (Choksi *et al*, 2006; Sun *et al*, 2003) with the following modifications. After genomic DNA extraction, non-sheared gDNA was verified on 1.5% agarose gel, and an overnight DpnI digestion reaction setup in a 50 µl reaction volume. The digestion reaction was subsequently purified using QIAGEN MiniElute PCR purified Kit and eluted in 50 µl MQ water. 50 µl of DpnI digested and purified DNA was further used for adaptor ligation. Adaptor ligated DNA was amplified using the adaptor specific primer to generate the TaDa-seq library. Amplified DNA from all experimental conditions was repurified (QIAGEN MiniElute PCR purification kit) into 20 µl of MQ water and 200 ng aliquots were run on 1% agarose gel to verify amplification of TaDa library (DNA fragments ranging from 500bp to 3Kb). The TaDa library was used for PCR-free library preparation followed by paired-end sequencing on a Illumina HiSeq 10x platform (BGI Tech Solutions, Hong Kong).

TaDa bioinformatics data analysis

Paired TaDa FASTQ sequences of the control sample with three biological replicates and dominant negative samples with two biological replicates (with two technical replicates for both control and dominant negative samples) were obtained for a total of 10 samples and used for subseqent analysis. After base quality assessment, reads were mapped to the reference genome of *Drosophila melanogaster* using Bowtie2 (-- very-sensitive-local) (Langmead & Salzberg, 2012) and post alignment processes were performed with sam tools and BED tools (Barnett *et al*, 2011; Quinlan, 2014). The *Drosophila melanogaster* reference sequence (FASTA) and gene annotation files were downloaded from Flybase and all GATC coordinates were extracted using fuzznuc (Rice *et al*, 2000) in BED format. Replicates were merged using Sambamba (merge) (Tarasov *et al*, 2015), and log-fold changes between control and dominant negative, obtained by deeptools bamCompare (--centerReads --scaleFactorsMethod readCount --effectiveGenomeSize 142573017 --smoothLength 5 -bs 1) (Ramirez *et al*, 2014) for BIGWIG (BW) file generation. Counts of reads mapped to GATC border fragments were generated using a perl script (GATC_mapper.pl) from DamID-Seq pipeline (Maksimov *et al*, 2016). GATC level counts were converted to gene level counts using Bedtools (intersectBed) (Quinlan, 2014). GATC sites were merged into peaks based on a previous study (Tosti et al., 2018). LogFC for individual GATC sites were generated using Limma for dominant negative vs control (P < 1e-5) and GATC sites were merged into peaks based on median GATC

fragment size in the *Drosophila* genome assembly using mergeWindows (tol=195, max.width=5000) and combineTests function from the csaw package (Lun & Smyth, 2016). Peaks were assigned to overlapping genes and filtered for FDR smaller than 0.05 and the mean logFC less than -2. All peak calling and statistical analysis was performed using the R programming environment. TaDa data can also be visualized using a custom UCSC (University of California, Santa Cruz) Genome Browser session (*https://genome-euro.ucsc.edu/s/vimalajeno/dm6*). WebGestaltR (Liao *et al*, 2019) was used for GO (Gene Ontology) for significantly downregulated TaDa candidates.

Integration of TaDa data with scRNA-seq and other omics data

Previously published wild-type third instar larval brain scRNA-Seq data (GSE198850) was employed (Pfeifer *et al*., 2022). Cellular heterogeneity was determined with eight different types of cells, including immature neurons, mature neurons, early neuroblast, NB-enriched cells, NB proliferating cells, optic lobe epithelium (OLE), Repo-positive cells and Wrapper-positive cells. The mature neuron population was divided into two groups for the current study: mature neurons and neuroendocrine cells. The neuroendocrine cell cluster was determined based on canonical markers (Guo *et al*., 2019; Huckesfeld *et al*., 2021; Nässel, 2018; Takeda & Suzuki, 2022; Torii, 2009). Subsequent analysis, including dimensionality reduction/projection or cluster visualization and marker identification was performed using R (Seurat) (Stuart *et al*, 2019) and Python (Scanpy) (Wolf *et al*, 2018) packages. Marker genes for each cluster were identified by FindAllMarkers function (Seurat) (Stuart *et al*., 2019). Clusters were visualized using two-dimensional Uniform Manifold Approximation and Projection (UMAP). The top 500 significantly downregulated genes from TaDa data (FDR<0.05 and mean logFC≤-2) were analysed in the third instar larval brain scRNAseq data. These 500 candidates were used as gene signatures, and enrichment analysis carried out using AUCell to determine whether a subset of the input gene set was enriched for each cell (with an enrichment threshold set at >0.196), and the clusters projected in UMAP based on the signature score (AUC score) (Aibar *et al*., 2017). Violin plots, dot plots, feature plots, heatmaps and matrix plots were plotted to visualize gene expression in the scRNAseq data. Functional enrichment analysis for the common significantly downregulated genes from the TaDa analysis was compared to neuroendocrine cell markers using WebGestaltR (Liao *et al*., 2019).

Circadian neuron scRNA-Seq data analysis

Publicly available circadian neuron scRNA-Seq data (10x) from the GEO database (GSE157504) was employed to investigate expression of *CG4577* in circadian neurons (Ma *et al*., 2021). The dataset d includes two conditions: LD (Light and Dark) and DD (Dark and Dark), as well as six time points: 2 hours, 6 hours, 10 hours, 14 hours, and 22 hours. After preprocessing, 3172 and 4269 cells remained for the LD and DD samples respectively, with a total of 15,743 and 15,461 RNA features. Subsequent analysis, including integration, dimensionality reduction/projection and cluster visualization was performed using R (Seurat) (Stuart *et al*., 2019). Based on clustering, 17 clusters were defined and visualized using two-dimensional Uniform Manifold Approximation and Projection (UMAP). Violin plots, dot plots, and feature plots were employed to visualize gene expression.

Immunohistochemistry

Relevant tissue (larval CNS or body wall muscle preparation) was dissected in cold PBS and tissues fixed in 4% formaldehyde at 4°C for 1 hour. Samples were washed 3 times with 0.1% PBS Triton X-100, followed by overnight incubation in 4% goat serum, 0.1% PBS Triton X-100. The following primary antibodies were used: guinea pig anti-Alk (1:1000, (Loren *et al*., 2003)), rabbit anti-Alk (1:1000, (Loren *et al*., 2003)), and rabbit anti-Dimm (1:1000 (Allan *et al*, 2005)), mouse mAb anti-PDF (1:1000, DSHB: C7), rabbit anti-Ilp2 (1:1000, (Veenstra *et al*, 2008)), anti-Dh44 (1:1000, (Cabrero *et al*, 2002)), rabbit anti-AstA (1:3000, (Stay *et al*, 1992) (Vitzthum *et al*, 1996), Jena

Bioscience GmbH), rabbit anti-Lk (1:1000, (Cantera & Nässel, 1992)), guinea pig anti-Spar (1:2000, this study), and Alexa Fluor®-conjugated secondary antibodies were from Jackson Immuno Research. Corrected total cell fluorescence (CTCF) was calculated using FIJI (Schindelin *et al*, 2012) by determining the level of cellular fluorescence and fluorescence intensity from microscopy images in third instar larval brains (n≥3 per genotype).

Immunoblotting

Third instar larval brains were dissected and lysed in cell lysis buffer (50 mM Tris-Cl, pH7.4, 250 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0,5% Triton X-100, complete protease inhibitor cocktail and PhosSTOP phosphatase inhibitor cocktail) on ice for 20 minutes prior to clarification by centrifugation at 14,000 rpm at 4°C for 15 minutes. Protein samples were then subjected to SDS-PAGE and immunoblotting analysis. Primary antibodies used were: guinea pig anti-Spar (1:1000) (this study) and anti tubulin (Cell Signaling #2125, 1:20,000). Secondary Antibodies used were: Peroxidase Affinipure Donkey Anti-Guinea Pig IgG (Jackson ImmunoResearch #706-035-148) and goat anti-rabbit IgG (Thermo Fisher Scientific # 32260, 1:5000).

Generation of anti-Spar antibodies

Polyclonal antibodies against Spar (CG4577) were custom generated in guinea pigs by Eurogentec. Two Spar peptides corresponding to epitopes LQEIDDYVPERRVSS (amino acids 212-226) and PVAERGSGYNGEKYF (amino acids 432-446) of Spar-PA were injected simultaneously.

Biochemical identification of Spar peptides

We re-examined peptidomic data from our previous study on the role of *Drosophila* carboxypeptidase D (SILVER) in neuropeptide processing (Pauls *et al*., 2019) for the occurrence of Spar. Peptides were extracted from brains from 5 d old male flies and analyzed on an Orbitrap Fusion mass spectrometer (Thermo Scientific) equipped with a PicoView ion source (New Objective) and coupled to an EASY-nLC 1000 system (Thermo Scientific) (for details see (Pauls *et al*., 2019)). Database search was performed against the UniProt Drosophila melanogaster database (UP000000803; 22070 protein entries) with PEAKS XPro 10.6 software (Bioinformatics solution) with the following parameters: peptide mass tolerance: 8 ppm, MS/MS mass tolerance: 0.02 Da, enzyme: “none”; variable modifications: Oxidation (M), Carbamidomethylation (C), Pyro-glu from Q, Amidation (peptide C-term). Results were filtered to 1% PSM-FDR.

CRISPR/Cas9 mediated generation of the *Spar$^{\Delta Exon1}$* mutant

The *Spar$^{\Delta Exon1}$* mutant was generated using CRISPR/Cas9 genome editing. Design and evaluation of CRISPR target sites was performed using the flyCRISPR Optimal Target Finder tool (Gratz et al., 2015). Single guide RNA (sgRNA) targeting sequences (sequences available in **Table S1**) were cloned into pU6-BbsI-chiRNA vector 2 (Addgene, Cat. No. 45946) and injected into *vasa-Cas9* (BDSC, #51323) embryos (BestGene Inc.). Injected flies were crossed to second chromosome balancer flies (BDSC, #9120) and their progeny were PCR-screened for progenitors carrying a 716 bp deletion event. Mutant candidates were confirmed by Sanger sequencing (Eurofins Genomics).

Generation of *UAS-Spar* fly lines

UAS-Spar was generated by cloning (GeneScript) the coding sequence of *CG4577-RA* into EcoRI/XbaI-cut *pUASTattB* vector followed by injection into fly embryos (BestGene Inc.) using attP1 (2nd chromosome, BDSC#8621) and attP2 (3rd chromosome, BDSC#8622) docking sites for phiC31 integrase-mediated transformation. Injected flies were crossed to second or third chromosome balancer flies, and transgenic progeny identified based on the presence of mini-white marker.

Measurement of Pupal size

Late pupae of the indicated genotype were collected and placed on glass slides with double-sided tape. Puparuim were imaged under Zeiss Axio Zoom.V16 stereo zoom microscope with a light-emitting diode ring light and measured using Zen Blue edition software. Both female and male pupae, picked randomly, were used for measurements.

Drosophila activity monitor assay

Up to 32 newly eclosed male flies were transferred into individual glass tubes containing food media (1% agar and 5% sucrose), which were each placed into a DAM2 *Drosophila* activity monitor (Trikinetics Inc). Monitors were then placed in a 25°C incubator running a 12:12h light:dark cycle, at a constant 60% humidity. Activity was detected by an infrared light beam emitted by the monitor across the center of each glass tube. The experiment was carried out for one month, and the raw binary data was acquired by the DAMSystem310 software (Trikinetics Inc.). The LD/DD experiment was performed according to previously published work (Chiu *et al*, 2010); adult flies were first entrained for 5 days in normal light:dark cycle and on the last day (LD5), the light parameters were switched off and flies were then conditioned in complete dark:dark settings for 7 days. Statistical and data analyses were carried out using Microsoft Office and GraphPad Prism 8.4.2, taking into consideration 5 min of inactivity as sleep and more than 24h of immobility as a death event. Actogram activity profile charts were generated using ActogramJ 1.0 (*https://bene51.github.io/ActogramJ/index.html*) and ImageJ software (*https://imagej.nih.gov/ij/*). ActogramJ was further used to generate the chi-square periodogram for each single fly in order to calculate the power value of rhythmicity and the percentage of rhythmic flies.

Data visualization and schematics

Schematics were generated at *Biorender.com (https://biorender.com/)* and *Bioicons.com (https://bioicons.com/)*. The pipeline icon by Simon Dürr *https://twitter.com/simonduerr* is licensed under CC0 *https://creativecommons.org/publicdomain/zero/1.0/* . Boxplots in **Figure 3D** and Suppl. Figure 7 were generated using BoxplotR (*http://shiny.chemgrid.org/boxplotr/*). Boxplots in **Figure 4** were generated using GraphPad Prism 9.

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Author contributions

S.K.S. and R.H.P. conceived the research. Wet lab experiments were conducted by S.K.S., L.M., J.S., G.U., P.M.-G., and T.M.. V.A. carried out all bioinformatics analysis. L.M. assisted with *Spar* mutant generation, validation and immunohistochemistry. J.S. performed and analyzed all *Drosophila* activity monitoring experiments under the supervision of M.S.. G.U. performed immunoblotting. P.M.-G. assisted in optimizing and performing TaDa experiments and T.M. performed image analysis and assisted with immunohistochemistry. A.S. and C.W. analyzed mass spectrometry peptidomic data. D.N. provided critical feedback and design input. R.H.P and M.S. supervised the project. S.K.S. and R.H.P. wrote the first manuscript draft that was further developed with all authors.

Competing interests

The authors declare that they have no competing interests

Data Availability Statement

The original contributions presented in the study are included in the article/Supplementary Material. The TaDa dataset has been deposited in Gene Expression Omnibus (GEO) under the accession number GSE229518. The genome browser tracks for the TaDa peak analysis can be found at: *https://genome-euro.ucsc.edu/s/vimalajeno/dm6*.

Supplementary figure legends

Supplementary Figure 1. TaDa third instar larval CNS sample validation and additional data analysis. A-B'. Expression of mCherry in the larval CNS reflects Dam-PolII expression. Third instar larval brains were stained for Alk (in green) and mCherry (in magenta) confirming expression of Dam-PolII in the TaDa system. Scale bars: 100 µm. **C.** Schematic overview of the TaDa analysis experimental workflow. Brains from third instar wandering larvae were dissected, and methylated DNA digested with Dpn1 restriction endonuclease. The resulting DNA fragment library was amplified, sequenced and analysed through TaDa bioinformatics pipelines. **D.** Bar graph showing total number of reads in each replicate of the TaDa dataset. **D'.** Bar graph showing percentage of reads aligned to *Drosophila* genome in each replicate. **E.** Correlation plot of samples (*control* and Alk^{DN}) and replicates shows no significant intra-replicate differences. **F.** Line graph indicating the relative distance to TSS of different samples compared to random regions. **G.** Pol ll occupancy profile of *Alk* in Alk^{DN} compared to control indicates a higher pol II occupancy in exons 1 to exon 7, in agreement with the expression of the Alk^{DN} transgene.

Supplementary Figure 2. Feature plots visualizing expression of TaDa-identified genes expressed in neuroendocrine cells in scRNA-seq from third instar larval CNS (Pfeifer *et al*., 2022). TaDa candidates *CG12594*, *cpx* and *VGlut* are shown.

Supplementary Figure 3. Co-expression of *Alk* and *Spar* in publicly available *Drosophila* CNS scRNA-seq datasets. UMAP showing co-expression of *Alk* and *CG4577* in different cell clusters in publicly available scRNA-seq data (Brunet Avalos *et al*., 2019) from first instar larval CNS **(A)** and **(B)** adult CNS (Davie *et al*., 2018). **C.** Pairwise alignment of CG4577-PA and CG4577-PB showing isoform-specific differences in amino acid positions 405 and 406 (highlighted in yellow).

Supplementary Figure 4. Alignment of *CG4577* orthologs in flies (Brachycera). **A.** Alignment of *Drosophila melanogaster CG4577* orthologs in the family *Drosophilidae* (vinegar flies, including the fruit fly *Drosophila melanogaster)*. **B.** Aligment of *Drosophila melanogaster CG4577* orthologs in other brachyceran taxa.

Supplementary Figure 5. A-A" Adult CNS showing Spar expression in Dimm positive cells. Spar (in magenta) and Dimm (in green), close-ups **(B-B")** indicated by boxed regions and arrows indicating representative co-expressed markers cells. Scale bars: 100 µm.

Supplementary Figure 6. *Spar* expression in adult neuropeptide expressing neuronal populations. **A.** Immunostaining of w^{1118} third adult CNS with anti-Spar (in magenta) and anti-PDF (in green). Closeups (**B-B")** of PDF- ans Spar-positive LNv neurons, indicated by white arrowheads. **C.** Immunostaining of w^{1118} adult CNS with Spar (in magenta) and Dh44 (in green). Closeups (**D-D")** of Dh44- and Spar-positiv e neurons, indicated by white arrowheads. **E.** Immunostaining of w^{1118} adult CNS with Spar (in magenta) and Ilp2 (in green). Closeups (**F-F")** showing the close proximity of Ilp2-positive and Spar-positive neurons in central brain, indicated by white arrowheads. **G.** Immunostaining of w^{1118} adult CNS with Spar (in magenta) and AstA (in green). Closeups (**H-H")** showing AstA- and Spar-positive neurons in central brain indicated by white arrowheads. Scale bars: 100µm.

Supplementary Figure 7. Spar does not affect the Alk-regulated pupal size phenotype. Overexpression of Spar (*C155-Gal4>UAS-Spar*) or *Spar RNAi* (*C155-Gal4>UAS-Spar RNAi*) in CNS does not significantly affect pupal size compared to previously characterized controls such as Alk^{DN} (*C155-Gal4>UAS-*Alk^{DN}), which significantly increase pupal size and overexpression of *Jeb* (*C155-Gal4>UAS-*Alk^{DN}), which significantly decrease pupal size compared to controls (*C155-Gal4>+*) (n.s = not significant, ***p*<0.05, ****p*<0.01).

Supplementary Figure 8. *Spar* expression in circadian neuronal clusters. A-B. Feature plots depicting the expression of *Spar* in publicly available circadian neuronal scRNA-seq data (Ma *et al*., 2021) throughout the LD cycle (Zeitgeber time) **(A)** and DD cycle (Circadian time) **(B)**. **C.** Dotplot showing *Spar* expression throughout the DD cycle along with the previously characterized circadian associated neuropeptide *Pdf* and the core clock gene *Per.* Peak expression of *Spar* and *Per* is observed at CT10.

Supplementary Figure 9. A. Representative activity profile graph of w^{1118} and $Spar^{\Delta Exon1}$ illustrating the average activity count measured every 5 min across a 24 hour span obtained by averaging 5 days in light/dark conditions (LD1-LD5). An unpaired student t-test was used to determine the significance between the two groups (****p<0.0001). **A'.** Graph illustrating the mean locomotor activity per day of w^{1118} and $Spar^{\Delta Exon1}$ obtained by averaging 5 days in light/dark conditions (LD1-LD5). An unpaired student t-test was used to determine the significance between the two groups (****p<0.0001). **B.** Representative activity profile graph of w^{1118} and $Spar^{\Delta Exon1}$ illustrating the average activity count measured every 5 min across a 24-hour span obtained by averaging 5 days in dark/dark conditions (DD1-DD5). CT0 and CT12 represent the start and end of the constant dark conditions respectively. An unpaired student t-test was used to determine the significance between the two groups (****p<0.0001). **B'.** Graph illustrating the mean locomotor activity per day of w^{1118} and $Spar^{\Delta Exon1}$ obtained by averaging 5 days in dark/dark conditions (DD1-DD5). An unpaired student t-test was used to determine the significance between the two groups (****p<0.0001). **C.** Representative sleep profile graph of w^{1118} and $Spar^{\Delta Exon1}$ illustrating the average activity count measured every 5 min across a 24-hour span obtained by averaging 5 days in light/dark conditions (LD1-LD5). An unpaired student t-test was used to determine the significance between the two groups (****p<0.0001). **E'.** Graph illustrating the mean sleep per day of $w^{1118\ and}$ $Spar^{\Delta Exon1}$ obtained by averaging 5 days in light/dark conditions (LD1-LD5). An unpaired student t-test was used to determine the significance between the two groups (****p<0.0001). **F.** Representative sleep profile graph of w^{1118} and $Spar^{\Delta Exon1}$ illustrating the

average activity count measured every 5 min across a 24-hour span obtained by averaging 5 days in dark/dark conditions (DD1-DD5). An unpaired student t-test was used to determine the significance between the two groups (****p<0.0001). **F'.** Graph illustrating the mean sleep per day of w^{1118} and $Spar^{\Delta Exon1}$ obtained by averaging 5 days in dark/dark conditions (DD1-DD5). An unpaired student t-test was used to determine the significance between the two groups (****p<0.0001).

Supplementary Figure 10. $Spar^{\Delta Exon1}$ flies retain a hyperactive profile when shifted to dark/dark conditions. A. Graph illustrating the Qp statistical value (rhythmicity power) obtained by generating the Chi-square periodograms of w^{1118}, $Spar^{\Delta Exon1}$, and $Alk^{\Delta RA}$ flies. An unpaired student t-test was used to determine the significance between wild-type and each mutant group (****p<0.0001). **A'.** Representative graph of the percentage of rhythmicity of w^{1118}, $Spar^{\Delta Exon1}$, and $Alk^{\Delta RA}$ flies. **B**. Graph illustrating the Qp statistical value obtained by generating the Chi square periodograms of w^{1118} flies in 5 days LD and 7 days DD conditions. A paired student t-test was used to determine the significance between the two experimental conditions (***p<0.001). **C**. Graph illustrating the Qp statistical value obtained by generating the Chi-square periodograms of $Spar^{\Delta Exon1}$ flies in 5 days LD and 7 days DD conditions. A paired student t-test was used to determine the significance between the two experimental conditions. **D**. Representative graph of the percentage of rhythmicity of w^{1118} flies in LD vs DD conditions. **E**. Representative graph of the percentage of rhythmicity of $Spar^{\Delta Exon1}$ flies in LD vs DD conditions.

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Editors

Reviewer #1 (Public Review):

Sukumar et al build on a body of work from the Palmer lab that seeks to unravel the transcriptional targets of Alk signaling (a receptor tyrosine kinase). Having uncovered its targets in the mesoderm in an earlier study, they seek to determine its targets in the central nervous system. To do this, they use Targeted DamID (TaDa) in the wild-type and Alk dominant negative background and identify about 1700 genes that might be under the control of Alk signalling. Using their earlier data and applying a set of criteria - upregulated in gain-of-Alk, downregulated in loss-of-Alk, and co-expressed with Alk positive cells in single cell datasets - they arrive upon a single gene, Sparkly, which is predicted to be a neuropeptide precursor.

They generate antibodies and mutants for Sparkly and determine that it is responsive to Alk signalling and is expressed in many neuroendocrine cells, as well as in clock neurons. Though the mutants survive, they have reduced lifespans and are hyperactive. In summary, the authors identify a previously unidentified transcriptional target of Alk signalling, which is likely cleaved into a neuropeptide and is involved in regulating circadian activity.

The data support claims made, are generally well presented and the manuscript clearly written. The link between circadian control of Alk signalling in Clock neurons > Spar expression > ultimately controlling circadian activity, however, was not clear.

Reviewer #2 (Public Review):

This manuscript illustrates the power of "combined" research, incorporating a range of tools, both old and new to answer a question. This thorough approach identifies a novel target in a well-established signalling pathway and characterises a new player in Drosophila CNS development.

Largely, the experiments are carried out with precision, meeting the aims of the project, and setting new targets for future research in the field. It was particularly refreshing to see the use of multi-omics data integration and Targeted DamID (TaDa) findings to triage scRNA-seq data. Some of the TaDa methodology was unorthodox (and should be justifed/caveats mentioned in the main text), however, this does not affect the main finding of the study.

Their discovery of Spar as a neuropeptide precursor downstream of Alk is novel, as well as its ability to regulate activity and circadian clock function in the fly. Spar was just one of the downstream factors identified from this study, therefore, the potential impact goes beyond this one Alk downstream effector.

Reviewer #3 (Public Review):

Summary:

The receptor tyrosine kinase Anaplastic Lymphoma Kinase (ALK) in humans is nervous system expressed and plays an important role as an oncogene. A number of groups have been signalling ALK signalling in flies to gain mechanistic insight into its various role. In flies, ALK plays a critical role in development, particularly embryonic development and axon targeting. In addition, ALK also was also shown to regulate adult functions including sleep and memory. In this manuscript, Sukumar et al., used a suite of molecular techniques to identify downstream targets of ALK signalling. They first used targeted DamID, a technique that involves a DNA methylase to RNA polymerase II, so that GATC sites in close proximity to PolII binding sites are marked. They performed these experiments in wild-type and ALK loss of function mutants (using an Alk dominant negative ALkDN), to identify Alk responsive loci. Comparing these loci with a larval single-cell RNAseq dataset identified neuroendocrine cells as an important site of Alk action. They further combined these TaDa hits with data from RNA seq in Alk Loss and Gain of Function manipulations to identify a single novel target of Alk signalling - a neuropeptide precursor they named Sparkly (Spar) for its expression pattern. They generated a mutant allele of Spar, raised an antibody against Spar, and characterised its expression pattern and mutant behavioural phenotypes including defects in sleep and circadian function.

Strengths:

The molecular biology experiments using TaDa and RNAseq were elegant and very convincing. The authors identified a novel gene they named Spar. They also generated a mutant allele of Spar (using CrisprCas technology) and raised an antibody against Spar. These experiments are lovely, and the reagents will be useful to the community. The paper is also well written, and the figures are very nicely laid out making the manuscript a pleasure to read.

Weaknesses:

My main concerns were around the genetics and behavioural characterisation which is incomplete. The authors generated a novel allele of Spar - Spar ΔExon1 and examined sleep and circadian phenotypes of this allele. However, they have only one mutant allele of Spar, and it doesn't appear as if this mutant was outcrossed, making it very difficult to rule out off-target effects. To make this data convincing, it would be better if the authors had a second allele, perhaps they could try RNAi?

Further, the sleep and circadian characterisation could be substantially improved. In Fig 8 E-F it appears as if sleep was averaged over 30 days! This is a little bizarre. They then bin the data as day 1 - 12 and 12-30. This is not terribly helpful either. Sleep in flies, as in humans, undergoes ontogenetic changes - sleep is high in young flies, stabilises between day 3-12, and shows defects by around 3 weeks of age (cf Shaw et al., 2000 PMID 10710313). The standard in

the sleep field is to average over 3 days or show one representative day. The authors should reanalyse their data as per this standard, and perhaps show data from 3-10 day old flies, and if they like from 20-30 day old flies. Further, sleep data is usually analysed and presented from lights on to lights on. This allows one to quantify important metrics of sleep consolidation including bout lengths in day and night, and sleep latency. These metrics are of great interest to the community and should be included.

The authors also claim there are defects in circadian anticipatory activity. However, these data, as presented are not solid to me. The standard in the field is to perform eduction analyses and quantify anticipatory activity e.g. using the method of Harrisingh et al. (PMID: 18003827). Further, circadian period could also be evaluated. There are several free software packages to perform these analyses so it should not be hard to do.